MEDICAL LABORATORY TECHNIQUES

FOR ROUTINE DIAGNOSTIC TESTS

Volume IX

MICROBIOLOGY

G. GURU
Project Coordinator

राष्ट्रीय शैक्षिक अनुसंधान और प्रशिक्षण परिषद्
NATIONAL COUNCIL OF EDUCATIONAL RESEARCH AND TRAINING

January 1991
Magha 1912

P.D. 5T - RA

© *National Council of Educational Research and Training, 1991*

ALL RIGHTS RESERVED

☐ No part of this publication may be reproduced, stored in a retrieval system or transmitted, in any form or by any means, electronic, mechanical photocopying, recording or otherwise without the prior permission of the publisher

☐ This book is sold subject to the condition that it shall not by way of trade, be lent, re-sold hired out or otherwise disposed of without the publisher s consent in any form of binding or cover other than that in which it is published

☐ The correct price of this publication is the price printed on this page Any revised price indicated by a rubber stamp or by a sticker or by any other means is incorrect and should be unacceptable

PUBLICATION TEAM

C N Rao *Head*, Publication Department

Prabhakar Dwivedi *Chief Editor*
Yacub Lakra *Editor*
Rekha Agarwal *Editorial Assistant*

U Prabhakar Rao *Chief Production Officer*
D Saiprasad *Production Officer*
C P Tandon *Art Officer*
Subodh Srivastava *Production Assistant*

Cover Design C P Tandon

Rs. 11,50

Published at the Publication Department by the Secretary National Council Of Educa- [illegible] New Delhi 110016, laser typeset at [illegible] 092 and printed at M/s Supreme [illegible] iya Nagar New Delhi 110 017
[illegible]

Foreword

The programme of vocationalization of higher secondary education has been accepted by the country as it holds forth great promise for linking education with the productivity and economic development of the country by providing education for better employability of the youth.

In view of the importance of the programme, the NCERT is making an all out effort to provide academic support to the implementing agencies in the States. One of the major contributions of the NCERT is in the field of curriculum development and in the development of model instructional materials. The materials are developed through workshops in which experts, subject specialists, employer's representatives, curriculum framers and teachers of the vocational course are involved.

The present volume on Microbiology is one of the series on "Medical Laboratory Techniques for Routine Diagnostic Tests" and is meant for students of the Medical Laboratory Technician course. It is being published for wider dissemination amongst students and teachers throughout the country. I hope that they will find the volume useful.

I am grateful to those who have contributed to the development of this volume. I must acknowledge also the immense interest taken by Prof. A.K. Mishra, Head, Department of Vocationalization of Education, in inspiring his colleagues in their endeavours to develop instructional materials. Shri G. Guru, Reader, functioned as the Project Coordinator for the development of this title. He has my appreciation and thanks for planning, designing and conducting the workshop, for technical editing and for seeing this title through the press.

Suggestions for improvement of this volume will be welcome.

K. GOPALAN
Director
National Council of
Educational Research and Training

New Delhi

Acknowledgements

The participation of the following experts in the development of this title as contributors is gratefully acknowledged.

Contributors

Dr. P.G. Sivananda
Dr. R.K Sharma
Dr S.K. Ghosh

Preface

Ever since the introduction of vocationalization of education in our school system by several States and Union Territories in our country, the paucity of appropriate instructional materials has been felt as one of the major constraints in the implementation of the programme and a source of great hardship to pupils offering vocational studies at the Higher Secondary stage

The Department of Vocationalization of Education of the National Council of Educational Research and Training, New Delhi, has started a modest programme of developing instructional materials of diverse types to fill up this void in all major areas of vocational education The task is too gigantic to be completed by any single agency but the model materials being developed by us might provide guidance and impetus to the authors and agencies desiring to contribute in this area These are based on the national guidelines developed by a working group of experts constituted by the NCERT

The present volume is on Microbiology and is meant for the pupils and teachers of the Medical Laboratory Technician vocation course being offered in a number of States and Union Territories It is hoped that the users will find it immensely useful

The material was developed during the workshop held in the Dr T M A Pai Research Centre, Manipal The present version was finalized after incorporating suggestions and comments The names of the contributors are mentioned elsewhere and their contributions are admirably acknowledged Shri G Guru, Reader and Coordinator of the project, Department of Vocationalization of Education, deserves special thanks for technical editing and for bringing the material into the present form The assistance of all in the Dr T M.A Pai Research Centre, Manipal especially of Dr. A. Krishna Rao, Dean, Kasturba Medical College, Manipal and that of the Department of Vocationalization of Education is also gratefully acknowledged

ARUN K. MISHRA
Professor and Head
Department of Vocationalization of
'Education

New Delhi

GANDHIJI'S TALISMAN

"I will give you a talisman.Whenever you are in doubt or when the self becomes too much with you, apply the following test

Recall the face of the poorest and the weakest man whom you may have seen and ask yourself if the step you contemplate is going to be of any use to him. Will he gain anything by it ? Will it restore him to a control over his own life and destiny ? In other words,will it lead to Swaraj for the hungry and spiritually starving millions ?

Then you will find your doubts and your self melting away"

MKGandhi

Contents

FOREWORD iii
ACKNOWLEDGEMENTS iv
PREFACE v

PART I INTRODUCTION

1.1 Definition and Scope of Microbiology 3
1.2 Microbes and Their Classification 5
1.3 Structure of Bacteria 9
1.4 Bacterial Nutrition and Growth Requirements 14
1.5 Bacterial Toxins and Enzymes 18
1.6 Bacterial Infection 20
1 7 Bacterial Studies 21

PART II BASIC LABORATORY REQUIREMENTS

2.1 "Do's and Don'ts" for the Technician 25
2.2 Glassware, Equipment, Containers and Chemicals 27
2.3 Sterilization 32
2.4 Microscopy 40
2.5 Culture Media, Their Preparation and Use 46
2.6 Collection of Clinical Materials for Microbiological Investigations 61

PART III LABORATORY TECHNIQUES FOR DETECTION AND IDENTIFICATION

3.1 Methods for Laboratory Investigation 71
3.2 Hanging Drop Preparation 77
3.3 Stained Preparation 80
3.4 Making a Sterile Transfer 89
3.5 Different Methods for Isolation of Bacteria 91
3.6 Culture of Various Clinical Specimens in the Laboratory 94
3.7 Anaerobic Cultivation 100

3.8 Water Analysis 103
3.9 Bacteriological Examination of Milk 105
3.10 Antibiotic Sensitivity Tests 108

PART IV IDENTIFICATION OF BACTERIA

4.1 Morphological Classification of Bacteria 113
4.2 Identification of Gram Positive Cocci 117
4.3 Identification of Gram Negative Cocci 122
4.4 Identification of Gram Positive Bacilli 124
4.5 Identification of Gram Negative Bacilli 130
4.6 Identification of Spirochaetes 138

APPENDIX

List of Contributors 139

PART I

INTRODUCTION

1.1 Definition and Scope of Microbiology

Microbiology is the "biology of small organisms" These organisms are too small to be seen by the naked eye. The limit of resolution with the unaided eye is about 200 microns Microorganisms being much smaller, can be visualized only under magnification, for which a microscope is required Microorganisms include viruses, bacteria, fungi, protozoa, and other parasites Most infectious diseases are caused by bacteria. So bacteriology (the biology of bacteria) was the first to be developed For this reason, previously the microbiology laboratory dealt mainly with bacteria and, to a less extent, with other pathogens. Gradually, the science of microbiology developed enormously and became subdivided into "bacteriology", "virology", "parasitology", "mycology", etc, for the sake of convenience. Presently, "bacteriology" has been retained as "microbiology" and the others have been dealt with separately. Again, in bacteriology, "anaerobic bacteriology" (dealing with obligate anaerobes), is coming up as a new section, separating the conventional aerobic or facultative ones.

These organisms are abundantly present just about everywhere in nature — in air, in water, in soil, in foods, in clothing, on and inside living bodies and all around us in the environment A small proportion of these microorganisms is disease-producing or pathogenic to human and animal life.

Medical microbiology is concerned with the causation or etiology, the mechanism of attack on tissue or pathogenesis, the laboratory diagnosis and treatment of disease in individuals infected by microorganism Microbiology also deals with epidemiology or the study of mass disease among people and the control or prevention of infection in the community.

Microbiology has a close link with curative medicine in regard to correct diagnosis, and in rational treatment of microbial diseases The doctor engaged in the cure of sick patients can often identify the pathogenic (disease-producing) microorganism from the typical clinical features of an infection and accordingly prescribe the appropriate treatment Sometimes, however, the patient has a fever but no characteristic signs or symptoms that will allow the doctor to make a precise diagnosis Even when the doctor identifies the disease from the patient's symptoms like sore throat, acute diarrhoea, pneumonia, meningitis, he will still need the help of the laboratory for accurate diagnosis because many of these symptoms are caused by different kinds of microorganisms For instance, acute diarrhoea may be due to some viruses, bacteria or protozoa Therefore, the treatment for the diarrhoea caused by these different microorganisms has to be different In other words, the doctor needs the laboratory to help establish the identity of the disease causing organism for the accurate treatment and cure of the patient

Knowledge of microbiology has an important role in the prevention and control of disease in the community Many diseases spread in the community by environmental and host factors e g overcrowding, contaminated food, drink, air, malnutrition, etc. The knowledge of these disease-causing microorganisms, their identification and mode of spread is, therefore, of prime importance for the control and prevention of disease in the community

The laboratory procedure in microbiology for isolation and identification of the causative agents is quite different from those of other clinical pathology laboratories. For instance, in diagnostic parasitology, the laboratory identification of parasites is based on microscopic observation of the active parasite or any component of its life cycle (tropozoites, cysts, eggs, larvae or adult segments) and culture of the parasite is not required. But in microbiology, which deals with diagnostic bacteriology, besides isolation and microscopic study, their culture, examination of biochemical characteristics, animal inoculation and serological tests, are most essential.

1.2 Microbes and Their Classification

The discovery of microbes led to the division of the living world into three kingdoms: the non-motile, photosynthetic plants; the motile, non-photo synthetic animals, and the third, the protist, distinguished from animals and plants by their relatively simple organization The microorganisms belong to this group of protista

The protists are subdivided into eukaryotes and prokaryotes In the eukaryotes (Gr. true nucleus) or higher protists, the cells are like those of plants or animals, contain a nucleus with a nuclear membrane, multiple chromosomes and mitotic apparatus Examples: protozca, fungi and most algae

The prokaryotes or the monera or lower protists. in these microorganisms there is, in fact, a single, naked chromosome, without a nuclear membrane and they lack other membrane bound organelles (e.g. mitochondria). Examples: Bacteria and blue green algae (cynabacteria), the precellular forms and the prokaryotes.

Table

Major groups of eukaryotic and prokaryotic organisms and viruses (simplified)

Eukaryotic cells	Algae Protozoa Fungi (including yeasts)
Prokaryotic cells	Bacteria Cyanobacteria Archaibacteria
Non-cellular, piece of nucleic acid protected by protein coat	Viruses

The viruses are not cells They are thought to be the linkage between the precellular forms and the prokaryotes

Among these major groups of organisms, bacteria, viruses and protozoa are the common causative agents of human infection. Recently, it has come to be known that some of the algae produce diseases in humans and animals. The cuanobacteria and Archaebacteria are of non-health importance.

Bacteria

Bacteria are small microorganisms with a prokaryotic form of cellular organization. Mostly they are unicellular. But cells may grow attached to one another in clusters, chains, rods, filaments, or, as in higher bacteria (Actinomycetales), a mycelium. Their cell size usually varies between 0.4 and 1.5 um (in short diameter), and in most cases they have a relatively rigid cell wall that maintains their characteristic shape.

Morphological study of bacteria is the most convenient way to classify them. Four major groups can be recognized on the basis of the mechanism of movement and the character of the cell wall: Gliding bacteria, Spirochaetes, Eubacteria, and Mycoplasmas.

Table
Key to the principal groups of bacteria

Principal Groups	*Genera that include species pathogenic to man*
I Flexible, thin-walled cells, motility conferred by gliding mechanism Gliding Bacteria	
II Flexible, thin-walled cells, motility conferred by axial filament Spirochaetes	Treponema Borrelia Leptospira
III Rigid, thick-walled cells, immotile or motility conferred by flagella: Eubacteria	
A Mycelial (actinomycetes)	Mycobacterium Actinomyces Nocardia Streptomyces
B. Simple unicellular	
1 Obligate intracellular parasites	Rickettsia Coxiella Chamydia
2 Free-living	
a Gram positive	Streptococcus
1 Good	Staphylococcus

2 Nonsporulating rods	Corynebacterium Listeria Erysipelothrix
3 Sporulating rods	
Obligate aerobes	Bacillus
Obligate anaerobes	Clostridium
b Gram negative	
1 Good	Neisseria
2 Nonsporulating rods	
Spiral forms	Spirillum
Straight, very small rods	Pasteurella Brucella Yersinia Francisella Haemophilus Bordetella
3 Enteric rods	
Faculative anaerobes	Escherichia (and related coliform bacteria) Salmonella Shigella Klebsiella Proteus Vibrio
Obligate aerobes	Pseudomonas
Obligate anaerobes	Bacteroides Fusobacterium
IV Lacking cell walls	Mycoplasma

Viruses

Viruses are classified as microorganisms, but they differ from all other forms of life The viral particle consists of a nucleic acid molecule, either DNA or RNA, enclosed in a protein coat called capsid The nucleic acid is infections. Viruses cannot multiply without living tissue. They require no food or water They do not release energy and they do not excrete waste They occupy a border area between living and non-living things. They are the smallest of microorganisms ranging in size from 20 to 300 millimicrons They can pass through a porcelain fitter that retains bacteria. Humans suffer from rabies, chicken pox, mumps, influenza, common cold due to the activities of viruses They are the major cause of diseases in all organisms.

Protozoa

Protozoa are unicellular, non photo-synthetic eukaryotes. They may be flagellated (Mastigophora) or non-flagellated. They are relatively large microorganisms, with transverse diameters mainly ranging from 2-100 n Most are free living, utilizing bacteria as their nutrient Others are saprophytic, deriving nourishment from dead plant or animal material The protozoa, producing disease are mainly parasitic, and derive their nutrition from the body of the host, e g , malaria parasite (plasmodium vivax) They reproduce by binary fission or multiple fission (schizogony), and some by a sexual mechanism Some of the protozoa have a thick walled resting stage or "cysts" which bypass the unfavourable environment

Fungi

The fungi are non-photosynthetic eukaryotes growing as a mass of branching, interlacing filaments ("hyphae") known as mycelium The mycelial forms are called moulds The yeasts are another type of fungus, which do not form a mycelium but are easily recognized as fungi by the nature of their sexual reproductive processes and by the presence of transitional forms Moulds reproduce by the formation of various kinds of sexual and asexual spores that develop from vegetative (feeding) mycelium or from aerial mycelium that affects their airborne dissemination.

These fungi are mostly saprophytic and are present in the environment They are mostly encountered as contaminants in the laboratory Several varieties of fungi occur naturally and can cause disease in man The most common of them is Candida Albicans It is normally present in the mouth, intestine and vagina It is responsible for infection in these sites when there is disturbance of local conditions or impairment of the defence mechanism Another common variety of fungal infection is Ringworm

1.3 Structure of Bacteria

1.3.1. Shape of Bacteria

Depending on the shape, bacteria are divided into several varieties

Cocci (Kokkos = berry) are spherical or oval cells.

Bacilli (baculus = rod) are rod-shaped cells

Vibrios are comma-shaped, curved rods and derive their name from their characteristic vibratory motility

Spirilla are rigid spiral forms

Spirochaetes (speira = coil, chaite = hair) are flexible spiral forms.

Actinomycetes are branching filamentous bacteria, so called because of a fancied resemblance to radiating sun-rays when seen in tissue lesions (actis = ray, mykes = fungus).

The above described shape of bacteria is due to the presence of their rigid cell wall.

Mycoplasmas are bacteria that are deficient of the cell wall and hence do not possess a stable morphology. They occur as round or oval bodies and as interlacing filaments. They are amorphous (without shape) bacteria

When the cell wall synthesis of bacteria is defective, either spontaneously or as a result of drugs that inhibit cell wall synthesis, e.g. penicillin, the bacteria lose their distinct shape. Such cells are called "protoplasts", or "L-forms"

1.3.2. Arrangement of Bacteria

Bacteria sometimes show a characteristic cellular arrangement or groupings.

Thus, cocci may be arranged in pairs (diplococci), chains (streptococci), group of four (tetrads) or eight (sarcina) or as grape-like clusters (staphylococci).

Some bacilli may be arranged in pairs (displobacilli), chains (streptobacilli), or arranged at angles to each other, presenting a cuneiform or Chinese letter pattern (corynebacteria)

The particular type of arrangement is a characteristic of the same bacterial group This is due to the tendency of the daughter cells to remain attached to parent cells even after division by repeated binary fission during their reproduction

1.3.3. Bacterial Structure

The structure of a typical bacterial cell is given in Fig 1.

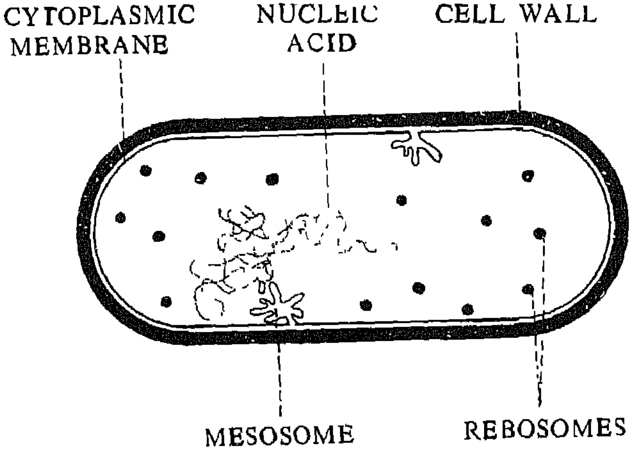


FIG 1 *Typical Bacterial Cell*

The protoplast or the living unit of the cell is surrounded by the thin, elastic, semi-permeable plasma membrane, which is covered by a rigid cell wall. The protoplast consists of cytoplasm, ribosomes, mesosomes, granules, vacuoles and nuclear body Some bacteria may contain additional structures in cytoplasm. The cell in some bacteria is enclosed by a viscid layer, which may be a loose-slime layer or organized as a capsule. Some bacteria may carry filamentous appendages coming out of the cell which may be flagella, the organ of locomotion, and fimbriae, the organ of adhesion.

Cell Wall

It is a rigid structure giving a particular shape to a bacterium It is about 10-25 mμ in thickness. Chemically two amino sugars (NAG and

NAM) N-acetylglucosamine and N-acetylmuramic acid enter into the composition of the wall. Gram positive bacteria contain teichoic acid The Gram negative bacteria contain lipo-polysaccharide Acid-fast bacilli contain mycolic acid Due to the above chemical nature, the bacteria differ in their staining property

Cytoplasmic Membrane

It is a thin (5-10 $m\mu$) limiting membrane of the cytoplasm It acts as a semi-permeable membrane and controls the inflow and outflow of metabolites to the protoplasm

Intracytoplasmic Inclusions

These inclusions are of a variety of types, and they act as storage granules.

Volution granules (metachromatic or Banes-Ernst granules) These are highly retractile, basophilic bodies comprising polymetaphosphate With toludin blue or methylene blue they stain metachromatically a red-violet colour, whereas the bacterial cytoplasm take a blue staining Special stains e g Albert's or Neisser's are used to demonstrate these Volution granules are characteristically found in diphtheria bacilli

Polysaccharide granules demonstrated by staining with iodine Lipid granules can be stained with fat soluble dyes e g sudan black

Nucleus

The bacterial nucleus is not surrounded by a nuclear membrane, so it is better called nuclear material It is a single long molecule of double stranded DNA (deoxy ribonucleic acid) present as a circle

Some bacteria may possess extra nuclear genetic materials consisting of DNA, which are called plasmids or episomes These plasmids or episomes confer on the bacteria certain properties e g drug resistance in the case of most enteric bacteria or toxigenicity in the case of diphtheria bacillus

Clime and Capsule

Many bacteria are surrounded by gelatinous material lying outside the cell wall When it is 0.2 μm thick it is seen in light microscope and called "capsule", when less than it is called "microcapsule", which is interpreted by electron microscopy. In most cases it is polysaccharide and in others it is polypeptide or protein. The capsular material confers virulence to certain microorganisms e g Pneuemococci

"Loose slime" or "free slime" is an amorphous material excreted by the bacteria into the surroundings. On solid media, these organisms produce "muciod" colonies which form a string when lifted by a wire loop. In liquid media, it is dispersed in the liquid.

Flagella

Flagella are the appendages for motility of different bacteria except spirochaetes. The flagellum is a long, thin filament, twisted spirally in an open regular wave-form. It is about 0.02 m. thick and may be several times longer than the bacteria Due to their thinness, these are not visible under a light microscope unless they are made thick by depositing different types of dyes over them or are seen under an electron microscope. Sometimes they can be seen under dark ground illumination Arrangement of flagella:

Peritrichous	— All round the cell
Polar	— At one or both ends
Monotrichous	— When the polar flagellum is single
Lophotrichous	— When the polar flagella are in tufts
Amphitrichous	— Flagella at both poles

Fimbriae or Pilli

Certain Gram negative bacilli carry fine hair-like appendages other than flagellae called "fimbriae or pilli". There are different types of fimbriae; classified according to hemaggulitination properties. The fimbriae may be "common" or "sex", depending on their function. The common fimbriae are the organ of adhesion by which the organism remains attached at the site of colonization or produces pathogenic effect. The sex fimbriae are the organ by which the extrachromosomal genetic bacteria are transferred from one cell to another, in certain cases, by way of conjugation

Spore

Some of the members of the Gram positive bacteria e.g. bacillus or clostridium species have the ability to form highly resistant resting stages called "spores" Each bacterium forms one spore in unfavourable conditions or on germination with favourable conditions, produces one vegetative cell These spores are called endospores as they are formed inside the bacterium.

The shape and position of the spores relative to the parent cell are characteristic to the organism. They may be central (equatorial), terminal or subterminal. They may be oval or spherical. They may or may not distend the bacterial body

These spores are extremely resistant to drying and, to a less extent, to chemicals and heat. The spores may resist boiling for a prolonged period, but medically important bacterial spores are destroyed by autoclaving at 121° C for 15 minutes.

Spores may be seen unstained in Gram's staining or can be stained by the modified Ziehl-Neelsen staining technique.

Pleomorphism and Involution Forms

Some species of bacteria exhibit great variation in their morphology during their process of growth, e.g swollen, spherical and pear-shaped forms, elongated filaments and filaments with localized swellings. This is called pleomorphism, and the cells are called involution forms. This is readily seen in plague bacilli. Involution forms may be obtained due to change in pH, and presence of cell wall synthesis-inhibitors

Spheroplasts and Free Protoplasts

When the bacteria are kept in sufficiently concentrated solutions e.g. 0.2-1 m sucrose with 0.01 Mg^{+2} and their cell wall is removed, they do not imbibe water or remain viable.

If the entire cell wall is removed, then they are called "free protoplasts" If there is some residual cell wall, then they are called "spheroplasts".

These forms are produced when the bacteria are grown in the presence or certain chemicals and antibiotics They are osmotically sensitive and lyse in the presence of diluted fluids.

L-forms of Bacteria

These are abnormal growth forms derived in the laboratory from bacteria of normal morphology, e.g. bacilli, cocci or vibrios These may be stable in the sense that when the agents e.g penicillin, etc. producing them are removed, they do not revert to normal form.

These L-forms are confused with the mycoplasmas. But these L-forms have antigenic similarity with the parent bacteria and differ from mycoplasma in their growth requirements.

L-forms could account for bacterial persistence in the human host suffering from diseases during therapy with antibiotics.

1.4 Bacterial Nutrition and Growth Requirements

The bacteria grow by a method of division which is called binary fission A new cell grows until it doubles its size and then it divides into two halves This can be done in the laboratory When the bacteria is introduced into a suitable artificial nutrient media and incubated under appropriate conditions, almost all the cells have the potential to grow at a very rapid rate

The physiology and biochemistry of bacteria are studied by observation made with culture grown in the laboratory in an artificial nutrient media. The growth and life of bacteria are influenced by various external factors such as nutrient materials in food, moisture, hydrogen-ion concentration, oxygen, carbon dioxide, temperature and light. These factors are discussed below

1.4. Bacterial Nutrition and Growth Requirements

Nutrition

The bacterial cell contains about 80% water, the rest comprises proteins, polysaccharides, lipids, nucleic acids, muco-peptides and low molecular weight compounds For growth, various organisms are adapted to different environmental conditions In the laboratory, it is very difficult to simulate the natural environment Though the chemical composition or metabolism of microorganisms resembles that of higher organisms, there are some individual differences, which are used in selective toxicity during their isolation or chemotherapy

Minimum nutritional requirements are water which acts as a vehicle for entry of substances into the cell and a source of H_2 and O_2, carbon, nitrogen and other inorganic salts

Bacteria can be classified based on their nutritional requirements and ability to synthesize their metabolites

Phototrophs:	Bacteria which derive their energy from sunlight.
Chemotrophs:	Those that derive their energy from chemical compounds.
Autotrophs	Bacteria that can synthesize all their organic compounds
Heterotrophs.	Those bacteria that cannot synthesize their own metabolites, but depend on preformed organic compounds

The pathogenic bacteria belong to the heterotroph group, and their nutrition requirements vary widely, as mentioned above. Their metabolites are to be supplied in the form of glucose, amino acids, nuclectides, lipids, carbohydrates and co-enzymes and inorganic salts

Oxygen Requirement and Metabolism

The bacteria can be classified into different groups depending on the effect of oxygen on their growth or viability

Obligate aerobes:	Grow only in the presence of oxygen, e g , pseudomonas sp.
Facultative anaerobes:	Can grow both in the presence or absence of oxygen Most of the clinical isolates belong to this group
Obligate anaerobes:	Growth occurs only in the absence of oxygen e g. clostridia sp
Micro aerophitic:	Grow better in the presence of low oxygen tension, e g., campylabacter sp

As mentioned above, for the isolation of a particular type of organism, oxygen tension of the culture environment in the laboratory should be favourable. The state of oxidation or reduction of a medium (or its environment) is called "redox potential", which is determined by the individual components of the medium, including its surroundings This can be measured as electrode potential or "En" or can be inferred by incorporating oxidation-reduction indicators e.g. methylene blue.

Carbon dioxide

All bacteria grow better in the presence of a small amount of carbon dioxide But Brucella sp organisms require about 10% carbon dioxide for their primary isolation

Temperature

For growth of bacteria, each species has its own range of temperature at which growth occurs best This is called "Optimum Temperature". Bacteria can be classified depending on the temperature of growth

Mesophilic:	Grow best between 25°C and 40°C The pathogenic organisms belong to this group, and their optimum temperature of growth is 37°C
Psychrophiles:	Grow below 28°C, even at 7°C, on unfrozen media These are nonpathogenic but cause food spoilage
Thermophiles	Grow best at 55°C to 80°C.

Heat is a good method of destruction of the microorganisms. Moist heat causes coagulation or dematuration of proteins, whereas dry heat causes oxidation and charring

At low temperatures, most organisms survive for a long time, but do not multiply. For this reason, domestic freezers are used to preserve different food preparations, so also the culture of different bacteria. Bacteria also can be preserved at –20°C to –78°C, in the presence of stabilizers such as glycerol

Moisture and Desiccation

As mentioned earlier, 80% of the bacterial mass consists of water Water is required for metabolism of other ingredients Thus, drying affects different organisms at varying time. *Treponema pallidum* (the causative agent of syphilis) is more susceptible, whereas staphylococci are more resistant to drying Bacterial spores may survive drying for a long time.

The vegetative, non-sporing organisms, if they are desiccated rapidly or completely while frozen and subsequently kept in a vacuum at room temperature, remain viable for appreciable periods This is called "eyophilization" and is a better method for preservation of bacteria

Influence of pH

A suitable hydrogen ion concentration (pH) is required for the growth of the bacteria Most pathogenic bacteria grow better at neutral or slight alkaline pH (7.2-7.6). Exceptions are *Vibrio cholerae*, resistant to alkaline but sensitive to acidic media

Influence of Light or Radiation

Darkness provides a favourable condition for the growth of bacteria (except photo-ophic) Ultraviolet light (from a mercury vapour lamp or sunlight) kills the bacteria rapidly, and is used as a method for sterilization in laboratories Bacteria are also killed by ionizing radiation Some bacteria may produce pigmentation after exposure to light and are known as photochromogens

Influence of Osmotic Pressure

Like other cells, bacteria are susceptible to variation in osmotic pressure, but are relatively resistant. For most species, the maximum concentration of sodium chloride permitting growth is between 5-12%. The organisms which can grow at higher concentration of salt (NaCl) are called halophilic (e g Vibrios)

Mechanical and Sonic Stresses

The bacterial cells are susceptible to vigorous shaking with fine glass beads or supersonic (9000-200,000 cycles/sec.) or ultrasonic (7200 000 cycles/sec.) vibrations This method is used to separate different components of a cell

1.5 Bacterial Toxins and Enzymes

The bacteria produces various substances while it grows in an animal body or in artificial media in the laboratory The nature of these substances may differ according to the conditions under which they are developed Some of these products of bacterial growth produce harmful effects in the animal or human tissue They are called bacterial poisons or toxins There are other products, the nature of which is that of enzymes or ferments.

This brings about important chemical changes in the surrounding media In some cases, the bacteria may produce pigments which give them their specific characteristics

· *Bacterial Toxins*

Toxins are defined as the products of bacteria which are injurious to the tissues of the host There are two types of toxins e.g exotoxins and endotoxins

Exotoxins An exotoxin is extracellular and is a specific injurious substance. This diffuses readily from the living bacteria into the surrounding medium. By removal of the bacteria the exotoxin can be separated, This can be done by centrifugation or by filtering through a Seitz filter The toxin remains in the supernatant fluid in the case of filtration. An example of exotoxin bacteria is *Corynebacterium diphtheria* Exotoxins are antigenic.

Endotoxins These are the poisons or toxins produced by bacteria and are intimately associated with the cell wall These toxins are retained within the bacterial cell until they die and disintegrate. The majority of disease-producing (pathogenic) bacteria produces endotoxins only Here the toxins are not liberated in the liquid medium, where the bacteria grow Hence, if a liquid culture containing such organisms is filtered through filters, the filtrate has little or no toxic effect, whereas the residue is very toxic

The differences between exotoxins and endotoxins are as below

Exotoxins	*Endotoxins*
1 Filterable	1 Not filterable
2 Usually fatal in small doses.	2 Large amounts are required to produce fatal effects
3 Thermostable	3 Relatively thermostable.
4. Capable of producing antitoxic serum.	4 Cannot produce antitoxic serum

Bacterial Enzymes

The bacteria sometimes produce certain special kinds of proteins acting as catalytic agents They are·

(a) *Proteolytic enzymes.* This enzyme is responsible for the decomposition of dead animal and vegetable matter in nature

(b) *Coagulase.* This is an enzyme which is often demonstrated during the study of biochemical properties of some pathogenic organisms.

(c) *Amylase·* This enzyme is capable of splitting carbohydrates, especially starch This is not used much in the study of bacteria

(d) *Lactic Acid Fermentation* They are also rarely studied for
(e) *Alcoholic Fermentation* biochemical analysis of bacteria

Bacterial Pigments

Many bacteria have the power to produce pigments. The pigments produced by different bacteria differ in colour and solubility e.g Staphylococcus amens produce golden yellow pigment and pseudonoma pyocyanens produce green pigment. Certain bacteria produce pigments which diffuse directly in the medium while others produce only pigmentation to the colony grown on the medium

1.6 Bacterial Infection

Infection is a process whereby the microorganisms or bacteria enter the animal or human body and produce disease. The various stages in the process are:

1. Entrance of the organism into the host through
   (a) respiratory tract,
   (b) digestive tract;
   (c) skin;
   (d) genito-urinary tract.
2. Establishment and multiplication of the organism within the host.

The organisms which produce disease in the host are known as pathogens. These pathogens, after entering the body of the host through different routes as mentioned above, reach the various systems of the body to produce disease. However, the mere presence of microorganisms in the body does not cause infection

The relationship between a pathogen and a disease is established by fulfilling the famous "Koch's postulates".

Koch's postulates:

1. The pathogen must be constantly found in the body of the host, either alive or dead
2. It must regularly be isolated and it must be grown in pure culture in *in vitro.*
3. When such a pure culture is inoculated into a sucesptible animal species, the typical disease must result.
4 From such experimentally induced disease, the pathogen must again be isolated.

1.7 Bacterial Studies

In bacteriological studies in the medical laboratory, microscopy is the primary base But direct examination of a bacterial smear is generally not sufficient to identify a bacterial species. Precise identification of the bacteria can only be obtained by a culture. For a culture of the bacteria, a well set up laboratory, with minimum requirements like sterilization facility, refrigeration and other allied inputs are essential

In the absence of such facilities, it is not possible to precisely identify the specimen. In such circumstances, it is very important to know the method for collection of a specimen and its despatch to a referral laboratory.

Nevertheless, the direct microscopical examination of stained smears is also an efficient way of studying the presence of bacteria in biology fluids that are normally sterile, such as cerebro-spinal fluid (C.S F) and pleural fluids and specimens from other sources. It may provide information of great value for the diagnosis, immediate treatment and control of the disease. Examples of such cases are.

1. In specimens from cases of male urethritis, an early stage of gonococcal infection can be diagnosed with reasonable certainty. (In females, of course, it is much more difficult).
2. Direct microscopy is believed to be a most practical and effective technique for the detection of infectious cases of tuberculosis and it has, therefore, great epidemiological importance.
3. Microscopic examination of stained smears of spinal fluid and study of morphology of any bacteria present may assist in identifying meningitis (meningococcal, pneumococcal or M. tubercular meningitis).

The study of the biochemical reactions of bacteria also helps in this identification

For understanding more clearly the nature, mechanism and cure of disease, the study of animal inoculations is undertaken

The diagnosis of some diseases is also possible through serology An example is syphilis Serological techniques are also important for epidemiological surveillance.

Thus, the general procedures for isolation, identification and study of bacteria are

1 morphology and staining reactions of the bacteria,
2. culture methods,
3. biochemical reactions,
4 animal inoculation,
5. serology

PART II

BASIC LABORATORY REQUIREMENTS

2.1 "Do's and Don'ts" for the Technician

The laboratory technician is called upon to play a vital role in the microbiology laboratory. He is assigned to undertake the investigation of the specimens as per request made by the clinicians. The duty demands his sincerity and promptness, and his ability to work with accuracy.

He is required to sterilize and maintain glassware, collect specimens, prepare smears for examination, prepare stained slides, prepare and maintain stock media, pour media in plates, inoculate specimens on various media and process tests for various isolated organisms, investigate cases of infection in hospitals, and perform other assigned duties

Before carrying out any laboratory test, the technician should be fully aware of the laboratory set-up, procedure and rules of conduct and strive to maintain a high standard. Before beginning work, he should take all precautionary measures and meticulously follow the sterile techniques always From the beginning, the technician should develop the habit of handling all cultures in the correct and safe manner, regarding them all as potentially infective.

Do's and Don'ts

1. Use an apron (white coat or gown) constantly to prevent contamination of your clothes.
2. Keep some extra clothes outside the laboratory
3. Keep the working place (work bench) neat and clean; do not keep unnecessary books and papers
4. Wash hand with running water and soap
5. Don't moisten labels, pencil, etc. with your tongue
6. Keep your fingers and hands away from your eyes, nose and mouth while you are working in the laboratory
7. Disinfect thoroughly or discard any personal article which

falls on the floor or on any other contaminated surface

8. Report all cuts, pricks, and abrasions, however slight, to the instructor or pathologist immediately
9. Do not remove any culture from the laboratory
10. Keep culture tubes upright in racks or containers, and be very careful that these are not ever laid on the bench
11. Keep the tubes and bottles always stoppered except when actually in use. Flame the lip of the tube or bottle immediately before and after use
12. Sterilize the inoculating loop thoroughly by heating until the wire is red hot before and after use Never put it down before sterilization.
13. Place the used slides and pipettes in jars containing disinfectant
14. Take care of your microscope after use, clean all the lenses with lens paper or clean cloth provided Clean the oil immersion lens carefully with a little Xylol.
15. Wipe off all water or other material from the stage and other parts
16. Leave your bench as you would like to find it Put all used slides, etc in the bowl of disinfectant, put the papers, etc in the bucket provided and not in the sinks
17. *Report immediately whenever you spill or splash infective material to your supervisor or pathologist immediately.*
18. Wash your hands thoroughly with soap and running water before you leave the laboratory.

2.2 Glassware, Equipment, Containers and Chemicals

2.2.1. Glassware

Glassware is one of the most important requirements of a microbiology laboratory The MLT is required to be familiar with the glassware, its maintenance, use and cleaning. The most commonly used glassware includes petridishes, test-tubes and pipettes of variable sizes Besides, there is other volumetric glassware and containers for chemicals and collection of samples. For details about glassware, their cleaning and maintenance, refer to Chapter-2, *Basic Laboratory Glassware, Equipment, Apparatus and their Maintenance, of Volume III, Laboratory Set-up and Procedure* of this series. Refer also to Chapter 2 1 on *Glassware of Volume VII, Clinical Biochemistry*

2.2.2. Equipment

Various types of equipment are used in a microbiology laboratory These include the autoclave, hot-air oven, incubator, inspissator, deionizer, refrigerator, centrifuge, distilled water plant, pipette washer, anaerobic jars, hydrogen and carbon dioxide cylinder, water bath, etc The students' microscope and analytical balance are of prime importance Refer to *Volume III Laboratory Set-up and Procedure and Volume VII Clinical Biochemistry* to know details about some of these equipment.

2.2.3. Containers for Collection of Clinical Specimens for Bacteriological Investigation

There are various types of containers for different kinds of clinical samples for specific bacteriological investigations.

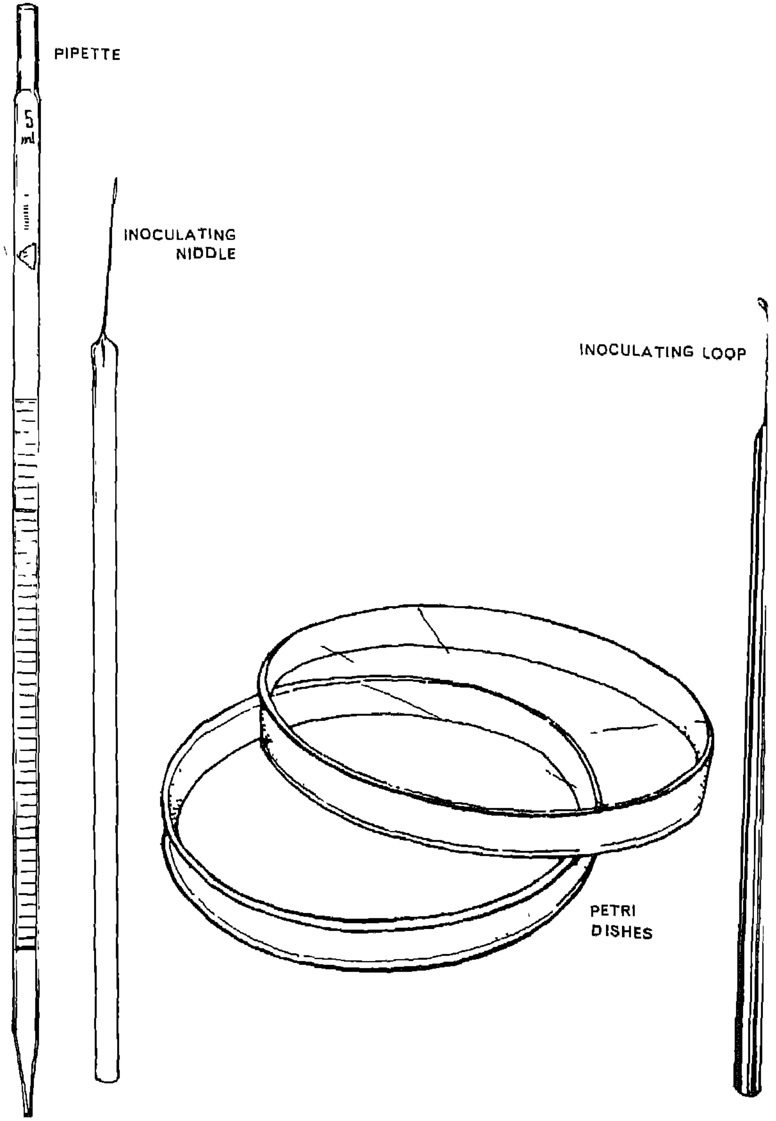


FIG 2 2 1 *Some Common Microbiology Laboratory Glassware and tools*

Refer to Chapter 6, *Specimen Containers*, of *Volume III Laborator Set-up and Procedure*, to know more about specimen containers

The types of containers used in the microbiology laboratory fo specific purposes are described below.

2.2.3.1 *Glass Tubes and Universal Containers*

These are suitable for submission of specimens of emulate pus, blood, cerebrospinal fluid, urine and faeces. Strong glass tubes, with rubber bungs or bark corks, should be used. These should be sterilized in the autoclave with the corks loosely fitted. Tubes with bark corks may be sterilized in the hot-air oven.

The screw-capped bottle known as universal container is recommended; it is a strong moulded glass bottle, with a flat base and wide mouth.

These bottles are sterilized by autoclaving with the caps loosely screwed on. They cannot be sterilized in the hot air oven, as the rubber washer will not stand the temperature.

The screw-capped universal container has many advantages over the glass tube. It is stronger, and the screw cap keeps the mouth of the container sterile, whereas dust collects at the rim of a stoppered tube. The contents of a universal container cannot leak or become contaminated and, as it is quite stable on its base, it is particularly convenient when specimens are taken at the bedside.

For the collection of serous fluids, e.g. pleural fluid, the universal container is suitable. The addition of 0.3 ml of a 20% solution of sodium citrate to the container prior to autoclaving (with the cap fitted) is recommended for the collection of fluids that may coagulate on standing. This helps to avoid difficulty in performing cell counts or centrifuging procedures with such fluids.

Blood can be collected in a universal container but for serological investigation, a small quantity of blood is more conveniently sent in a sterile glass tube fitted with a rubber bung. The blood clot may be cultured in a selective medium e.g. for enteric organism (Typhoid). Blood intended primarily for blood culture should be submitted in a special blood culture bottle.

For the collection of faeces, a small squat bottle or glass specimen tube fitted with a bark cork in which a small metal spoon is fixed, is used. But such containers have disadvantages because on removal of the cap, some of the specimen may be withdrawn and get contaminated.

For small quantities of faeces, the universal container is normally recommended. A small spoon made of tin plate can be included in the container prior to sterilization. A portion of faeces is taken up in the spoon and the whole dropped into the container and the cap screwed on.

A screw capped jar with a wide mouth, known as a "Promade Pot" is also used and here the faeces can be taken up in small card-board spoons (such as are used for ice cream cartons).

These containers can also be transmitted to the laboratory by post In such cases, the package should be labelled "pathological specimen"

In case there is likely to be a delay of some hours before laboratory cultivation can be carried out, neutral laboratory cultivation can be carried out, neutral glycerol saline should be added to the faeces

For small quantities of urine, e g for the diagnosis of most cases of urinary tract infection, the universal containers are used For large quantities of urine e.g complete early morning specimens, screw-capped bottle are convenient

Special care must be taken for a urinary specimen for culture It should be sent to the laboratory as soon as possible after the specimen has been collected In women, preliminary cleaning of the external genitalia should always be insisted upon, and in all cases the mid-stream specimen is the best.

2 2 3 2 Disposable Containers

Besides the above type of containers, disposable containers are now being used for bacteriological laboratory specimens These are

(a) *Aluminium containers* (5 cm diameter and 2 5 cm in depth). These are suitable for the collection of faeces or sputum. Such containers can be dispatched through the post

(b) *Plastic containers* These are made of polystyrene and/or polypropylene They are sterilized and may be with or without a spoon These containers are made in a variety of sizes and are suitable for urine, faeces, pus, blood, etc Special precautions are to be taken for transporting 'high risk' specimens like blood examination for *hepatis virus B.*

(c) Disposable glass containers are also used and they are now being manufactured in the country.

All disposable containers should be destroyed in a furnace or fire after use.

2 2.3 3 Swabs

A swab usually consists of a piece of aluminium or tinned iron wire 15 gauge, 15 cm long One end is made rough for about 0 5 cm by squeezing it in a small metal vice or cutting pliers Around this end a thin pledget of absorbent cotton wool is lightly wrapped for about 2 cm. The wire is placed in a narrow thick walled test-tube 12 5 cm X 1.25 cm and the lip of the tube is plugged with cotton wool.

When the swabs are to be sent by post or otherwise to a distant laboratory, the wire should be 12 cm and the top inserted into a bark cork, which stoppers the tube The tube with the swab should be sterilized in the autoclave and not in the hot air oven, because in the hot air oven, the wool may char. It is important to autoclave cork-stoppered tubes with the cork loose and to press the cork after sterilization Tubes plugged with cotton wool should be dried after autoclaving.

Instead of wire, swabs can be prepared from wooden sticks, 16 cm long, that are specially made for the purpose and are known as "peerless wooden application " A cottonwool pledget is wrapped around one end as described earlier, and the tube is plugged with cotton wool These cannot be used easily with a bark cork It has the advantage that the stick can be broken off short, when the swab needs to be placed for transportation in a screw-capped container

The swab may also be purchased loose and unsterile for assembly and sterilization in tubes This type of swab is useful in the laboratory for other purposes e.g, seeding of media for the disk sensitivity test.

Swabs are very useful for taking specimens from (a) throat, (b) wounds, discharging ears or surgical conditions like fistula, sinus, etc ; (c) post-nasal or nasopharyngeal space, (d) rectum, (e) cervix of the uterus.

While taking specimens from babies and young children, special precautions are to be taken. It is necessary to employ a very fine swab

In cases where there is a possible time lag between swab collection and examination, it is advisable to place the swab in transport medium. This is specially important for delicate organisms like meningococcus

2.2.4. Chemicals

Grades of chemicals, common chemicals and their preservation, and, preparation of saturated, percent, molar, molal and normal solutions are described in detail in *Chapter 2 3 Chemical and Reagents* of *Volume VII Clinical Biochemistry*

Chemicals required in the microbiology laboratory include (i) those which are used as ingredients for the preparation of different kinds of culture media, (ii) different dyes, and (iii) sterilizing agents

2.3 Sterilization

2.3.1. Introduction

In the practice of medicine and surgery, specially for practical microbiology, the procedures that kill the microorganisms have an important application.

This is due to the fact that a scientific study of bacteria or microorganisms in the laboratory, the containers or culture media should be free from other microorganisms present in the environment as well as in the laboratory equipment It is important that all the laboratory containers, glassware and appliances be made free from all live, contaminated microorganisms for proper identification and diagnosis of the particular disease-producing bacteria. At the same time, it is also necessary to avoid infecting the patients from any disease-producing organism through instruments, dressings, nursing equipment and from drugs which are administered to the patients through injections.

The procedures to kill or remove the microorganisms are described as sterilization and disinfection. It is important to recognize the difference between these two procedures Sterilization is an absolute term which indicates complete killing or removal of microorganisms of all kinds, whereas disinfection is a relative term and means a degree of removal of pathogenic (disease-producing) microorganisms.

Definition of Sterilization

Sterilization means freeing of an article from all living organisms, including viruses, bacteria and their spores, and fungi and their spores, both pathogenic and non-pathogenic. Sterility is an absolute state. An article should never be described as being "relatively sterile" — it is either sterile or unsterile.

Uses

Sterilization is required for culture media, suspending fluids, reagents, containers and equipment used in microbiology It is also required for medical and surgical instruments and materials used in procedures that involve penetration into the blood, tissues and other parts of the body e g in surgical operations, infusions, hypodermic infection and diagnostic aspirations, etc

Techniques of Sterilization and Disinfection

Sterilization and disinfection can be carried out through (a) physical agents — drying, freezing, radiation, heat, filtration, and (b) chemical agents

2.3.2. Drying

Drying kills many bacteria, but many pathogens such as the tubercle bacilli, staphylococcus aureus and the small pox virus can survive in dust for long periods

2.3.3. Freezing

Cold increases the viscosity of proteins, slows down the streaming action of protoplasm and kills the bacteria. If the bacteria are in a liquid medium and frozen slowly, they can be possibly damaged by ice crystals

The process of lyphylization, a combination of freezing and drying is a satisfactory method for long term storage of bacterial culture In this process, culture or bacterial suspensions are rapidly frozen and then evaporated to dryness in high vacuum Ampules containing the lyphylized organisms are sealed and then can be stored at room temperature

2.3.4. Sterilization by Heat

Sterilization by heat is of two types — by moist heat and by dry heat. Moist heat is much more effective than dry heat. Moist heat kills microorganisms by coagulation and denaturing their enzymes and structural proteins, whereas dry heat is believed to kill microorganisms by causing a destructive oxidation of essential cell constituents.

The factors to be considered for sterilizing by heat are the temperature and time of exposure, the number of vegetative microorganisms and spores present and also the material containing the microorganisms

The following minimal sterilizing exposure should be followed, in relation to the temperature

Moist Heat		Dry Heat	
Temperature	Sterilizing time	Temperature	Sterilization time
100°	20 hours	120°	8 hours
110°	2 1/2 hours	140°	2 1/2 hours
115°	50 minutes	160°	1 hour
121°	15 minutes	170°	40 minutes
125°	6 1/2 minutes	180°	20 minutes
130°	2 1/2 minutes		

2 3.4.1 Sterilization by dry heat

1. *Flaming:* Direct exposure for a few seconds in a gas or spirit flame may be used for sterilizing the mouths of flasks and culture tubes, slides, scalpels and needles, but it is uncertain, because only the surface actually touched by the flame is sterilized

2 *Red Heat* Platinum loops, inoculating wire, points of forceps, needles are sterilized by holding them in the flame of a Bunsen burner until they are observed to be red hot.

3. *Hot Air.* This is the main means of sterilization by dry heat. Hot air at 160°C for one hour will destroy all bacteria and their spores, and is used for sterilizing all dry glassware such as test-tubes, petridishes, flasks, pipettes, throat swabs, syringes, instruments, needles.

For this, a hot air oven is used The oven is usually heated by electricity and has a thermostat that maintains the chamber air constantly at the chosen temperature of 160°C and a fan to assist in circulation of air.

The glassware should be perfectly dry before being placed in the oven. The instrument to be sterilized should be wrapped in craft paper and placed in such a manner as to allow free circulation of air in between. After the oven is loaded it is heated upto the sterilizing temperature The holding time of 1 hour at 160°C is required from the time when the thermometer shows 160°C and this temperature is to be maintained for 1 hour. The oven should be allowed to cool for about an hour before the door is opened, since the glassware may get cracked by sudden cooling

4 *Infra-red Radiation.* This is another method of sterilization by dry heat employing infra-red radiation. The infra-red rays are directed from an electrically heated element on to the objects to be sterilized, e.g all glass syringes, at a temperature of 180°C

Surgical instruments are sterilized at or above 200°C by infra- red rays in vacuum.

2.3.4.2 Sterilization by Moist Heat

Moist heat kills spores and bacteria quicker than dry heat. Killing by moist heat requires the microorganisms to be in contact with hot water or steam If the articles for sterilization are protected from getting wet by grease or are in a sealed impervious container, they will be subject only to the weaker effect of dry heat at room temperature

Moist heat is used for culture media, liquids, glass with rubber or metals parts, filters, infected materials, surgical gloves, dressing linen, etc

Moist heat may be employed (i) at temperature below 100°C, (ii) at a temperature of 100°C i e. in boiling water or free steam, and (iii) at temperature above 100°C i.e in saturated steam under increased pressure in an autoclave

The third procedure ensures sterilization and killing of highly resistant spores

1 *Moist heat at temperature below 100°C.* In pasteurization of milk, the temperature employed is either 63°C to 66°C for 30 minutes and this process usually destroys all non- spore forming pathogens, such as mycobacterium tuberculosis, mycobovies, brucella and various salmonellae that may be present in milk.

Vaccines prepared from pure cultures of non-sporing bacteria may be inactivated in a special water bath (vaccine bath) at a comparatively low temperature; 1 hour at 60°C is usually sufficient. Higher temperature may diminish the immunizing power of the vaccine

Eating utensils, clothes, bedclothes and some items of nursing equipment are disinfected by washing in water for several minutes at 70°C to 80°C

2. *Moist heat at temperature of 100°C or boiling at 100°C* Boiling at 100°C for 5-10 minutes is sufficient to kill all non- sporing and many sporing bacteria Some spores don't get killed even on boiling for more than one hour

Sterilization by exposure to steam (generated at atmospheric pressure) is also carried out occasionally. It can be done in an autoclave without clamping down the lid in a Koch's steamer

(Koch's steamer is a vertical metal cylinder with a conical lid It is fitted with a thermometer and has a small opening for steam to escape For steam to generate, water is poured into the cylinder Above the water level there are one or two perforated shelves to pass the steam).

Sterilization by free steam is carried out by,

(i) prolonged exposure for $1\frac{1}{2}$ hour — used for broth and nutrient agar.

(ii) intermittent exposure at 100°C for 20 to 45 minutes on each of successive days. The method is used for culture media containing sugar that are decomposed at higher temperature and for gelatin media which after prolonged heating fail to solidify on cooling

The principle of this method is that the first heating kills vegetative forms of bacteria and, between heating, the spores, being in a favourable medium, germinate and become vegetative forms, which in turn get destroyed on the subsequent heating.

3. *Moist heat at temperature above 100°C.* The sterilization by this method is done by an autoclave

Principle of autoclave

When water is boiled in a closed container, it produces saturated steam under pressure, the temperature of which will rise above 100°C

This is the principle employed in the pressure cooker and the autoclave which subjects the article for sterilization to moist heat at a temperature higher than 100° All types of microorganisms are killed when the apparatus is heated for 20 minutes at 120°C in the steam under pressure. Autoclaving is the method most widely used for sterilization of surgical supplies and bacteriological culture media.

The simple form of a laboratory autoclave is the 'pressure cooker' type It consists of a vertical or horizontal cylinder of gun metal or stainless steel in a supporting frame or case The size may be upto about 45 cm in diameter and 75 cm in length. The cylinder contains water upto a certain level and this is heated by a gas burner or electric heater below the cylinder. The lid or the door is fastened by screw clamps fitted with an asbestos gasket to make it airtight. At the top of the autoclave there is a discharge tap, a pressure gauge and a safety valve. The discharge tap is kept open for few minutes after the water begins to boil to allow all the air in the chamber to be discharged in the stream of steam. The tap is then closed and then the pressure in the chamber rises to the desired pressure for autoclaving This is normally $15\frac{1}{2}$ lb/per square inch The autoclaving is continued thus for the time set The safety valve is to be set to release steam at the chosen pressure, which is automatically maintained throughout the period of sterilization.

After the set time, the source of heat is removed and the pressure is allowed to be released. (If an autoclave is opened while still under positive pressure, an explosion may occur, leading to an accident). As soon as the pressure falls, the autoclave should be opened cautiously. If it is allowed to cool without opening the lid, after the pressure falls, there will be excessive boiling and evaporation of aqueous media.

Based on this principle, modern autoclaves with automatic process and sterilization indicators have been developed

Steps to use the autoclave

- *Preparation of material for sterilization.* The preparation of material differs from item to item.

For syringes. These are placed in large glass tubes plugged with non-absorbant cottonwool (the pistons and barrels are to be separated and kept in separate tubes) or they are wrapped in gauze or cotton wrappers and placed in metal trays.

Needles Needles should be placed separately in small test-tubes that are then plugged or in the syringe wrapper, in a different bunch Place a pad of non-absorbant cotton wool at the bottom of each tube to protect the tips of the needles

Needles can also be arranged in a metal tray with the points stuck into a folded gauze pad.

Glassware Specimen tubes, petridishes are normally wrapped in brown paper and tied with string.

Pasteur pipettes These are placed in large tubes and then plugged They can also be wrapped in brown paper

Sterilization procedures

- Fill the bottom of the autoclave with water upto the inner support of the wire gauge or basket
- Place the basket containing the material to be sterilized in the autoclave.
- Close the lid or door tightly
- Open the air discharge tap
- Begin heating the autoclave
- Watch the air discharge tap for continuous uniform steam to come out
- Observe the gauge, when the desired temperature is reached (i.e. 120°C), the heat should be regulated and maintained.
- Timing for autoclaving should be:
  - (a) Syringes, needles, tubes, etc. for collecting specimens — 20 minutes at 120°C
  - (b) Containers for infected material like sputum pots, pus tubes,

etc. — 30 minutes at 120°C

(c) Bacteriological culture media as per requirement of different culture media

- Turn off the heat as soon as the required time is over
- When the temperature falls below 100°C, open the air discharge tap to equalize the pressure with the outside atmosphere.
- Take off the lid, as soon as the hissing sound of the air inlet taps stops

The same method can be used for sterilization in a pressure cooker also.

2.3.4.3 Sterilization by Radiation

Ultra-violet rays, which are available in sunlight are also used for sterilization. Ultra-violet rays from suitably shielded lamps have been used to reduce the number of air-borne bacteria. Such lamps are fitted in laboratories or operation theatre in which highly infected material is handled.

Ionizing radiation Ionizing radiation and X-rays and gamma rays are used nowadays to sterilize disposable plastic articles like syringes, I.V sets, etc.

2 3.4 4 Sterilization by filtration

In bacteriological practice, sterilization by filtration is very commonly used, specially when it is undesirable for the material to be subjected to heat or chemicals

The method comprises passing the fluids, including the bacterial culture, through special filters with a pore size of 0 75 μ.

The method is especially useful for sterilization of various solutions such as media containing carbohydrates, serum and antibiotic solutions

Various types of filters are used in a microbiology laboratory The most common ones are

— Seitz Filter (asbestos and asbestos paper discs),

— Berkefeld Filter (infusorial earth candle shaped filter);

— Sintered Glass Filter,

— Cellulose membrane filters.

The Seitz filter has the disadvantage of being adsorptive The membrane filters are non-adsorptive and have a much higher flow rate than others The additional advantage is that the bacteria retained on their surface can be stained and counted

2.3.4.5. Chemical Sterilization

Chemical antimicrobial agents are used in killing or inhibiting many pathogenic microbacteria. In actual terms, they cannot be relied on to kill all microorganisms and spores — they are either disinfectants or antiseptics.

The disinfectants are potent microbicidals but are toxic substances. They rapidly kill the vegetative forms of pathogenic organisms but are ineffective against spores. They are not used on living tissues. The antiseptics are non-toxic and used for disinfection of living tissues (e.g. body surface).

The antibacterial effects of chemical agents may be bactericidal (killing effect) or bacteriostatic (growth inhibiting effect). Only a few of the more toxic and irritant disinfectants such as formaldehyde, glutaraldehyde and ethylene oxide are capable of killing bacterial endospores, and they are effective only when used in adequate concentration applied under carefully controlled conditions of temperature, moisture, etc.

2.4 Microscopy

2.4.1. Microscopy

Microbiological study requires the use of microscope as microbes are invisible to the naked eye. A microscope is essentially an optical instrument consisting of a lens or combination of lenses used to magnify minute objects or microorganisms which cannot be seen by the naked eye. Microscopy has come a long way since Leeuwenhoek first observed the world of 'little animalcules' in 1683 using hand ground lenses. Now various types of microscopes are employed.

2.4.2. Types of Microscopes

There are various types of microscopes.

1. Simple Microscope
2. Compound Microscope
3. Electron Microscope
4. Phase contrast Microscope
5. Ultra-violet Microscope
6. Fluorescent Microscope
7. Polarizing Microscope

A simple microscope consists merely of a single magnifying lens and produces a virtual, erect and magnified image, when an object is in its focal length.

The *common microscope* that is suited for the routine study of microorganisms is the *typical compound microscope*, either monocular or binocular. This consists of two converging lenses fixed at the ends of a brass tube. The lens nearer to the object is called the objective and the lens through which the final image is observed is called the eye-piece The objective magnifies the specimen to a definite degree and produces a real, inverted intermediate image of the object, which lies within the principal focus of the eye-piece The eye-piece further magnifies the image formed by the objective so that the image seen by the eye has a magnification equal to the product of magnification of two systems. The individual magnification of the objectives and eye-piece is engraved on each part The final image seen is, thus, inverted, magnified and virtual

2.4.3. Parts of Compound Microscope

It consists of

1 *Mechanical Parts:*
   - Stand
   - Limb
   - Stage, etc
2 *Magnifying Parts.* Optical lens system
   - — Objectives
   - — Eye-pieces
3. *Illuminating Parts.*
   - Substage condenser
   - Iris diaphragm
   - Filter holder
   - Mirror

2 4.3.1. Mechanical Parts

(a) *Stand and Limb:* It forms the base of the microscope. It comprises a heavy foot, often horse-shoe shaped to give stability, the limb which bears the illuminating apparatus, the stage and the observation tube The limb is attached to the foot by a hinged joint so that the microscope can be set at a comfortable angle for the observer.

(b) *Stage:* The stage is a platform which accommodates a glass, a microscope slide on which the object to be examined is mounted. It has an aperture in its centre to permit light to reach the object. The stage can be of two types

(i) A fixed stage, in which the object is fixed by clips

(ii) A mechanical stage, in which the object can be moved to a desirable distance either sideways or forward and backward by the rack and pinion mechanism. This type of stage is preferable when a large area of the microscopic preparation has to be searched as in the examination of sputum smears for tubercle bacilli or blood smears for malarial parasites

2.4.3 2. Magnifying Parts

This consists of the eye-pieces and objectives. They are kept

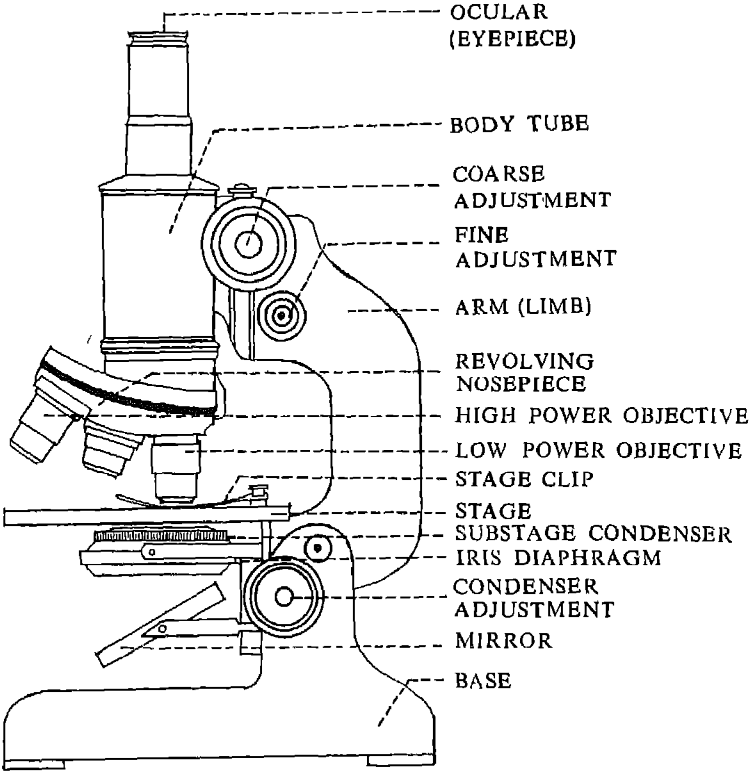


FIG 2 4 3 *Compound Microscope*

separated in a graduated tube. These are focussed by the rack and pinion mechanism for both coarse and fine adjustments. The objectives are screwed onto the revolving nose-piece, which is attached to the lower end of the tube. They are usually three in number and are designated according to their focal lengths.

(i) Low power objective (16 mm)
(ii) High power objective (4 mm)
(iii) Higher power oil immersion objective (2 mm)

The eye-pieces (oculars) are usually designated by their magnification like 5x, 6x, 10x, 15x, etc.

2.4.3.3. Illuminating Parts

This consists of the following parts:

(a) *Substage condenser*
It is made up of a system of convex lenses, which focus light from the illuminating source on the plane of the object The height of the condenser and, therefore, the focus of light, can be varied at will by a rack and pinion mechanism Lowering of the condenser diminishes the illumination whereas raising it increases the illumination While using the oil-immersion objective, the condensor is completely raised as it requires more light. When the other objectives are used, it is lowered suitably.

(b) *Iris diaphragm*
Immediately below the condenser and incorporated in the same mount is the substage iris diaphragm operated by small lever which protrudes to one side. Opening or closing of this iris diaphragm controls the amount of light reaching the object. The iris diaphragm is opened widely when the oil-immersion objective is used as it requires maximum light, and is closed partially when the other objectives are in use

(c) *Filter holder*
Just below the iris diaphragm is a ring-shaped filter holder designated to carry circular, coloured glass filters required to reduce the excessive red or yellow component of some light sources.

(d) *Mirror:*
Fitted to the tail-piece, below the condenser, is a plano-concave mirror. It helps to reflect the light into the substage condenser. The flat surface is used whenever the oil-immersion objective is employed. The concave mirror is employed with low and high power objectives.

2.4.4. Magnification

It is defined as the degree of enlargement of the image of the object achieved by the microscope.

The magnification of a microscope is the product of the separate magnifications of the objective and the eye-piece.

(a) Low power objective magnification 10 X 10 = 100 times
(b) High power objective magnification 40 X 10 = 400 times
(c) Oil immersion objective magnification 80 X 10 = 800 times
or
100 X 10 = 1000 times

2.4.5. Use of Microscope

Adjust the various parts as follows.

1. *For Unstained Preparations*
   (a) Lower the condenser
   (b) Close the iris diaphragm
   (c) Use concave mirror
   (d) Focus under low power and then turn to high power

2 *For Stained Preparations*
   (a) Lower the condenser
   (b) Use concave mirror
   (c) Adjust the iris diaphragm to give even illumination of field
   (d) Focus under low power and then turn to high power, if required.

3 *For Oil-immersion Examination*
   (a) Raise the condenser completely
   (b) Open the iris diaphragm
   (c) Use plane mirror
   (d) Place a drop of cedar wood oil on the object and focus under oil immersion lens (100x) Rack oil immersion objective down till its tip dips into the oil Using the fine adjustment, focus the object.
   (e) After use, remove the oil from the objective and the object, with a cloth dipped in xylol
   (f) Leave low power objective in position for further use

2.4.6. Care of Microscope

Clean the microscope with a clean, soft cloth The objectives and eye-pieces must be cleaned with lens paper and xylol. Alcohol should not be used as it dissolves the cement which binds the lenses Set up the microscope in a convenient position facing the source of light. Place the object on the stage and adjust the mirror to the illuminant. Direct sunlight on the mirror should be avoided For prolonged work, artificial light of particular wave lengths is advisable

When not in use, protect the microscope from dust and damage

2.4.7. Microbiological Unit of Measurement and Microscopy

The unit of measurement used in microbiology is the micron (micrometre) which is one thousandth of a millimetre.

1 micron (μ)

$= \frac{1}{1000}$ millimetre (mm)

= 1 micrometre (μm)

= 1000 millimicron (mμ)

= 1000 nanometre (nm)

= 10,000 Angstrom unit (Å)

The limit of resolution with the unaided eye is about 200 micron (μ) or micrometre (μm). Microbes being much smaller can be visualized under magnification Bacteria of medical importance generally measure 0.2 to 1.5 μ in diameter and about 3 to 5 μ in length.

The resolving power of the light microscope is limited by the wave length of light With visible light the limit of resolution of a microscope, using the best optical system is about 300 nm. The resolving power of an electron microscope, in practice, is about 0.1 nm or 1 Å

4.6 Identification of Spirochaetes

Spirochaetes are spiral, elongated, motile, flexible organism The three important genera are *Treponema, Leptospira* and *Borrelia*

Morphology

They are exceedingly delicate, spiral filaments. 6-14 μ by 0.13 μ with six to twelve coils which are comparatively small, strong and regular In the unstained condition the demonstration requires dark field illumination *T pallidium* cannot be demonstrated by the ordinary staining method. The organism may be demonstrated by Fontana's method or the India Ink method, using the exudate from the chancre

Pathogenicity

T. pallidium is the causative organism of syphilis.

Diagnosis

It can be diagnosed by a serological test

2.5 Culture Media, Their Preparation and Use

Artificial culture media constitute the food materials for the cultivation of microorganisms *in vitro* The culture media are used to propagate microorganisms so that they can be isolated in pure form from various infected materials like blood, urine, pus, sputum, etc Culturing helps to study the colonial characteristics and various other properties of different bacteria All the *in vivo* composition and conditions of the tissues and body fluids must be provided in these culture media for the successful growth of bacteria *in vitro*.

2.5.1. Basic Requirements of Culture Media

1. Nutrients.

Energy source — Derived from oxidation and decomposition of proteins and carbohydrates

Carbon source — Ex: Citrate

Nitrogen source — Ex: Peptone

2 Mineral salts like sulphates, phosphates, chlorides, carbonates of sodium, potassium, magnesium and calcium. Usually NaCl is added for the growth of parasitic organisms to bring the osmotic pressure up to a figure comparable with that of host tissues

3 A suitable pH, usually between 7 2 and 7 6

4. Necessary growth factors Ex. (a) Tryptophan for *S. typhi* growth (b) X & V factors for the cultivation of Haemophilus influenzae.

2.5.2. Classification of Culture Media

There are many systems of classifying culture media

I. Depending upon the known and unknown composition of ingredients:
   - Synthetic media, where the exact composition of all in-

  gredients will be known
  - Non-synthetic media, where the exact composition will not be known

II. Depending on the consistency
  - Liquid media
  - Semisolid media
  - Solid media

III. Depending upon the oxygen requirement of organisms:

Aerobic media
  1. Simple media
  2. Enriched media
  3. Enrichment media
  4. Differential or Indicator media
  5. Selective media
  6. Sugar media
  7 Transport media

Anaerobic media

2.5.3. Simple Media

These media consist of only the basic necessities for growth of the non-fastidious organisms. These media can either be solid or liquid

Ex. 1 Nutrient broth (Peptone + Meat extract + NaCl + D water)
2. Nutrient agar (N. broth + agar)
3. Peptone water.

(1) *Nutrient broth.*

This is the basis of most media used in the study of the common pathogenic bacteria.

Preparation

Peptone - 10 gms
Meat extract (Lab-hemCo) - 10 gms
NaCl - 5 gms
Distilled water - 1 litre

Mix the ingredients and dissolve them by heating briefly in the steamer (pressure cooker). When cool, adjust the pH to 7.5-7 6 A precipitate of phosphates may appear and this may be removed by filtration through a filter paper. The presence of a deposit of phosphates does not interfere with the nutrient value of the medium but it may hinder the recognition of slight bacterial growth indicated turbidity

Distribute the broth in tubes and sterilize by autoclaving at 121°C for 15 minutes

Identification· Yellow coloured liquid kept in tubes or bottles.

Uses:

(1) It acts as a basis for the preparation of various solid media

(2) To study the growth curve

(2) *Nutrient agar*

Preparation· Nutrient broth + 2% agar

Heat the ingredients in a steamer to dissolve the agar Sterilize by autoclaving at 121°C for 15 minutes After sterilization allow the media to cool to 50°C and then pour it into tubes (as slopes or as stand) or petridishes

Identification. Yellow coloured solid media in tubes or plates.

Uses·

(1) Used for the growth of non-fastidious organisms.

(2) For testing antibiotic sensitivity

(3) Nutrient agar slopes are used to maintain stock cultures

(4) Pigment producing organisms are better appreciated by a nutrient agar plates. Ex. Pseudomonas aeruginosa

(3) *Peptone water*

Preparation·

Peptone	-	10 gms
NaCl	-	5 gms
Distilled water	-	1 litre

Adjust the pH to 7 5-7 6 Dispense in bottles or tubes and sterilize by autoclaving at 121°C for 15 minutes

Identification A watery liquid kept in test-tubes or bottles

Uses

(1) It acts as a simple media for the growth of non-fastidious organisms

(2) To test the Indole production.

(3) Preparation of sugar media for biochemical tests

(4) For enrichment of certain organisms
Ex Alkaline peptone water for *Vibrio cholerae*

2.5.4. Enriched Media

These are the simple media containing certain additional substances like blood, serum or egg. These media are used to cultivate fastidious bacteria which are more exacting in their nutritional requirement.

Ex 1. Serum agar (Nutrient agar with 5-10% serum)
2 Blood agar (Nutrient agar with 5-10% blood)

3 Chocolate agar (Blood agar heated to 70°C)
4. Loeffler's serum slope

(1) *Blood agar*

Preparation Add 5-10% sterile blood (either human or animal) to sterile nutrient agar that has been melted and cooled to 50°C Dispense in petridishes

Identification Blood coloured medium in petridishes when viewed through transmitted light is almost opaque.

Uses.

(1) Used for the growth of fastidious organisms like gonococcus, Haemophilic group of bacteria, etc
(2) It is also an indicator medium showing the haemolytic properties of bacteria such as Streptococcus pyngenes

(2) *Chocolate agar* (Heated blood agar)

Preparation Melt the nutrient agar, cool it to 75°C, add the blood and allow the medium to remain at 75°C, mixing the blood and agar gently from time to time until the blood becomes chocolate brown in colour within about 10 minutes. Then pour it into petridishes.

An alternative method is to keep the blood agar plates in an incubator or hot air oven at 55°C for 1-2 hours

Identification· Chocolate coloured opaque medium in plates

Uses

Used in culturing pneumococci, gonococci, meningococci and Haemophillus group of organisms

(3) *Serum agar*

Preparation· Add 10% sterile serum to sterile nutrient agar that has been melted and cooled to 55°C Dispense in plates or tubes.

Identification Pale yellow coloured medium in plates or tubes

Uses· Used in culturing more exacting organisms.

(4) *Loeffler's serum slope*

Preparation

Sterile ox, sheep or horse serum	-	300 ml
Nutrient broth	-	100 ml
Glucose	-	1gm

Dissolve the glucose in the broth and autoclave at 115°C for 20 minutes Add the glucose broth to the serum with sterile precautions and distribute in sterile test-tubes or in 2 5 ml amounts in 1/4 of sterile bottles The tubes/bottles are placed in the sloped tray of the inspissator and the temperature is slowly raised to 80-85°C and maintained for 2 hours. When the serum coagulates to a yellowish white solid inspissated medium, it should be allowed to cool before being handled.

Identification Yellowish-white solid slopes in tubes or bottles

Uses

(1) Used for the cultivation of corynekacterium diphtheriane The diphtheria bacilli grow luxuriently producing a large amount of metachromatic granules which helps in their identification

(2) Used to demonstrate the proteolytic properties particularly of the Clostridium species.

2.5.5. Enrichment Media

These media are incorporated with certain chemical substances which enhance the growth of pathogenic organisms and suppress the unwanted bacteria The enrichment media prevent the non-pathogenic or commensal bacteria from over-growing the pathogenic organisms

Ex: (1) Tetrathionate broth For Typhoid - para-
(2) Selanite F broth typhoid bacilli
(3) Alkaline peptone water for *Vibrio cholerae*

(1) *Tetrathionate broth*

Preparation

(a) *Thiosulphate solution:*

Sodium thiosulphate	- 24.8 gms
Distilled water	- 100 ml

Mix the salt and water with sterile precautions and steam at 100°C for 30 minutes

(b) *Iodine solution*

Potassium iodide (KI)	- 20 gms
Iodine (I)	- 12.7 gms
Distilled water	- 100 ml

Dissolve KI in about 50 ml of warm water, add the iodine and make up to a final volume of 100 ml

Preparation of complete medium

Calcium carbonate	- 2.5 gms
Nutrient broth	- 78 ml
Thiosulphate solution	- 15 ml
Iodine solution	- 4 ml
Phenol red, 0.02% in 20% ethanol	- 3 ml

Add the calcium carbonate to the broth and sterilize it by autoclaving at 121°C for 15 minutes. When cool, add the thiosulphate, iodine and phenol red solutions with sterile precautions Distribute in 10 ml amounts in sterile screw-capped bottles.

Identification Peptone water-like liquid in screw-capped bottle

Uses Used to selectively grow typhoid - paratyphoid group of organisms from faeces, suppressing the growth of Coliform bacilli (Tetrathionate inhibits the growth of Coliform bacilli)

(2) *Selanite F broth*

Preparation.

Sodium acid selanite - 4 gms
Peptone - 5 gms
Lactose - 4 gms
Disodium hydrogen phosphate - 9 5 gms
Sodium dihydrogen phosphate - 0 5 gms
Sterile water - 1 litre
pH of the medium - 7 1

Dissolve the ingredients with sterile precautions and distribute the solutions in 10 ml amounts in sterile screw-capped bottles Steam at 100°C for 30 minutes

Identification Yellow solution in screw-capped bottles

Uses. Selenite inhibits Coliform bacilli while permitting bacilli of the typhoid - paratyphoid to grow freely.

(3) *Alkaline Peptone water (APW)*

Preparation Adjust the pH of the peptone water to 8 2-9.2

Uses Encourages the growth of *Vibrio cholerae*, while inhibiting the Coliform bacilli in faeces

Identification Same colour as that of peptone water distributed in screw-capped bottles.

2.5.5. Differential (Indicator) Media

The best example is McConkey's medium

Preparation of Mc Conkey's medium

Peptone - 20 gms
Sodium taurocholate - 5 g
Distilled water - 1 litre
Agar - 20 gms
Neutral red solution, 2% in 50% ethanol - 3 5 ml
Lactose 10% aqueous solution - 100 ml

Dissolve the peptone and taurocholate in the water by heating Add the agar and dissolve it in a pressure cooker Adjust the pH to 7.5 Add the lactose and neutral red, which should be shaken well before use and mix Heat in the pressure cooker with free steam and then sterilize at 115°C for 15 minutes Pour it into plates

Identification: Distinct reddish brown transparent media in plates.

Uses: Used as a differential medium for Gram negative bacilli into lactose fermenters and non-lactose fermenters.

The organisms which can attack lactose will ferment it, producing acid. This acid production will be indicated by change in colour of the neutral red indicator. As a result, the colonies of these organisms will appear pink in colour and these are called *LACTOSE FERMENTERS*. Ex. *E coli, Klebsiella, Citrobactor* These bacteria which ferment lactose, but are late in action, will develop light pink colonies. Ex: Shigella sennoi. These are called *LATE LACTOSE FERMENTRS.* Those bacteria which do not ferment lactose at all will develop pale or almost colourless colonies. These are known as *NON-LACTOSE FERMENTERS* Ex. *Pseudomonas, Proteus, Salmonella, Vibrio,* etc

2.5.6. Selective Media

Selective media serve the same purpose as enrichment media But these are solid media containing the inhibitory substance

Ex: 1 Deoxycholate citrate agar for dysentery bacilli
2. Wilson and Blair's media for *Salmonella typhi*
3. McLeon's potassium tellurite media for diphtheria bacilli
4. Lowenstein-Jensen's medium for mycobacteria.

(1) *Deoxycholate citrate agar* (DCA)

Preparation.

(a) *Neutral red lactose agar*

Meat extract (Lab - Lemco) - 20 gms
Peptone - 20 gms
Agar - 90 gms
Neutral red solution (2% in 50% ethanol) - 5 ml
Lactose - 40 gms
Water - 4 litre

Dissolve the meat extract in 200 ml water over a flame Make just alkaline to phenolphthalein with 50% NaOH (i.e. adjust the pH to 10), boil at 100°C and filter Adjust the pH to 7.4; make up the volume to 200 ml and add the peptone. Dissolve the agar in 3800 ml water by steaming at 100°C for 1 hour Add the meat extract-peptone solution and mix and then add neutral red and lactose, mixing again Pour it into 400 ml lots and sterilize at 115°C for 15 minutes.

(b) *Solution A*

Sodium citrate (Analar) - 17 gms
Sodium thiosulphate (Analar) - 17 gms

Ferric ammonium citrate - 4 gms
Sterile distilled water - 100 ml

(c) *Solution B*

Sodium deoxycholate - 10 gms
Sterile distilled water - 100 ml

Prepare solution 'A' and solution 'B' with sterile precautions, heating at 60°C for 1 hour

(d) *Preparation of complete medium*

Neutral red lactose agar - 400 ml
Solution A - 20 ml
Solution B - 20 ml

Melt the neutral red lactose agar and add solutions A and B in this order, using separate sterile pipettes and mix well in between Pour into plates immediately.

Identification Pale pink coloured medium in plates

Uses.

(1) Used for the isolation of *Shigella* sp, *Salmonella paratyphi B* and the salmonella food poisoning group
(2) It is found to be superior to Mc Conkey's medium, but is not so selective for *S. typhi*.

Bile salts will inhibit the growth of many Gram positive organisms Neutral red is included as an indicator Sodium citrate and sodium thiosulphate are toxic to coliforms and to a certain extent to *Salmonella* sp Ferric citrate is added to neutralize this toxicity for *Salmonella* without interfering too much with the toxicity to coliforms

Lactose fermenting coliforms appear as pink columns with precipitation of deoxycholate surrounding the colony Non-lactose fermenter (ex. *Sh. sonnei*) becomes pale pink on further incubation. *Salmonella* sp form as colourless colonies with a black central dot, which is due to H_2S production which in turn combines with ferric citrate to give iron sulphide

(2) *Wilson and Blair's media for Salmonella typhi*

Preparation·

(a) *Bismuth sulphite glucose phosphate mixture*

Bismuth ammonium citrate - 30 gms
Sodium sulphate - 100 gms
Disodium hydrogen phosphate - 100 gms
Glucose - 50 gms
Sterile distilled water - 1 litre

With sterile precautions, dissolve the bismuth ammonium citrate in 250 ml boiling water and the sodium sulphate in 500 ml boiling water. Mix the solution and while the mixture is boiling, add the sodium phosphate crystals. When this mixture is cool, add the glucose dissolved in 250 ml boiled and cooled water.

(b) *Iron citrate brilliant green mixture*

Ferric citrate - 2 gms
Brilliant green - 0 25 gms
Sterile distilled water - 225 ml

With sterile precautions, mix solutions of the ferric citrate in 200 ml water and the brilliant green in 25 ml water

(c) *Preparation of complete medium*

Sterile 3% nutrient agar - 100 ml
Bismuth sulphite glucose phosphate mixture - 20 ml
Iron-citrate brilliant green mixture - 4.5 ml

Melt the agar and cool to 60°C. Add the other ingredients with sterile precautions and pour into plates

Identification· Pale green opaque medium in plates.

Uses:

(1) Selective medium for *Salmonella typhi* and *Salmonella paratyphi B*

The sodium sulphate in the medium is reduced to sulphide in the presence of glucose by these organism, yielding black columns E.coli is inhibited by brilliant green and bismuth sulphite in the presence of excess sodium sulphite

(3) *McLeon's potassium tellurite media for diphtheria bacilli*

Preparation:

Nutrient agar - 100 ml
Sterile defilumated blood - 10 ml
Sterile potassium tellurite solution 4% aqueous - 1 ml

Melt the nutrient agar and when the temperature has come down to 15°C, add the blood and tellurite solution, with sterile precautions Pour into plates.

Identification: Chocolate brown coloured medium in petridishes.

Uses· Used in the isolation and typing of conjne bacterium diphtheriae on colonial morphology. The colonies appears black in colour. Potassium tellurite inhibits most pathogenic or commensal species of the throat other than conjnebacterium diphtheriae

(4) *Lowenstein - Jensen's medium for mycobacteria*

Preparation

(a) *Mineral salt solution*

Potassium dihydrogen phosphate (anhydrous) - 2.4 gms

Magnesium sulphate - 0.24 gms
Magnesium citrate - 0.6 gms
Asparagine - 3.6 gms
Glycerol - 12 ml
Distilled water - 600 ml

Dissolve the ingredients bv heating Autoclave at 121°C for 15 minutes to sterilize.

(b) *Malachite green solution*

Malachite green - 2gms
Sterile distilled water - 100 ml

Dissolve the dye in sterile distilled water by keeping it in the incubator for 1-2 hours

(c) *Preparation of complete medium*

Mineral salt solution' - 600 ml
Malachite green solution - 20 ml
Beaten egg (20 to 22 hen's eggs) - 1 litre

All glassware used to prepare the complete medium must be sterile The eggs must be fresh (not more than 4 days old). Wash the eggs throughly with a brush and soap and rinse them well in running water Drain off the water and dry the eggs properly Before handling the clean, dry eggs, scrub the hands and dry them Crack the eggs with a sterile knife into a sterile, wide-mouthed bottle with glass beads Stopper and shake the bottle up and down Measure 1 litre of this beaten egg and add to the complete medium Mix well.

Distribute the complete medium in 5 ml amounts in 1 oz Mc Cartney bottles and screw the caps on tightly Lay the bottles horizontally in the inspissator and heat at 75-80°C for 1 hour After solidification, remove the bottles and store in the refrigerator.

Identification. Light green slopes in 1 oz Mc Cartney bottles

Uses

(1) Used for the isolation of *Mycobacterium tuberculosis.* Colonial morphology on this medium allows the differentiation of human and other types

(2) The growth of the human type is enhanced by glycerol. Malachite green suppresses the growth of organisms other than Mycobacteria

(3) Used to check the drug sensitivity of *Mycobacterium tuberculosis.*

2.5.7. Sugar Media

The term "SUGAR" refers to any fermentable substance like carbohydrates, glucosides, etc Different sugars like glucose, lactose, mannital, sucrose, etc. are used to study the biochemical reactions of various organisms, which will enable identification of the genus and species of various bacteria.

The sugar media contain 1% desired sugar in peptone water and an appropriate indicator like Andradc's indicator. A small tube called a DURHAM'S TUBE will be kept inverted in the sugar for the detection of gas production. If the sugar is fermented, the colour of the medium will turn pink. Gas formation is detected by the accumulation of gas in the Durham's tube.

For studying the biochemical reactions of fastidious organisms like pneumococci or diphtheria bacilli, HISS SERUM SUGARS are used, which contain an additional 3% serum.

2.5.8. Transport Media

Transport media arc specially used whenever there is delay in the transportation of the specimen to the laboratory, for instance, while transporting the specimen containing delicate organisms like Gonococci. These media are also used to prevent the pathogens being overgrown by commensals. i.e. in the transportation of dysentery or cholera stool specimens.

Ex: 1. Stuart's medium for *Gonococci*
2. Sach's buffered glycerol saline for *Salmonella*
3. Cary-Blair's medium for *V. cholerae*
4. Venkatraman and Ramakrishnan's media for *V. cholerae*

(1) *Stuart's medium for Gonococci*

Preparation

(a) *Anaerobic salt solution*

Thioglycollic acid - 2 ml
NaOH 1N - 12-15 ml
Sodium glycerophosphate 20% aqueous - 100 ml
Calcium chloride 1% aqueous - 20 ml
Distilled water (free of chlorine) - 900 ml

Mix the ingredients, adding sufficient NaOH to bring the pH to 7.2

(b) *Agar solution*

Agar - 6 gms
Distilled water - 1 litre

Dissolve by steaming.

(c) *Preparation of complete medium*

Anaerobic salt solution - 900 ml

Agar solution - 1 litre
Methylene blue 0.1% aqueous - 4 ml

Melt the agar and add the salt solution Adjust the pH to 7.3- 7 4. Add the methylene blue and distribute in bottles, filling nearly up to the brim. Autoclave at 121°C for 15 minutes and immediately tighten the caps; when cool the medium should be colourless. If the medium is blue, before use, heat the bottles in a pressure cooker (free steam) so as to drive out the dissolved oxygen in the medium and once again the medium will become colourless.

Identification: Colourless semi-solid media in Bijin bottles.

Uses:

(1) Used to maintain the gonococci and Bordetella pertusis on swabs during their transmission

(2) Recovery of Shigella from rectal swabs

(2) *Sachs' buffered glycerol saline for Salmonella*

Preparation

Sodium chloride - 4.2 gms
Potassium dihydrophosphate - 1 gm
Potassium orthophosphate - 3.1 gms
Phenol red - 0.003 gms
Distilled water - 700 ml
Glycerol - 300 ml

Adjust the final pH to 7.2.

Dispense in 10 ml amounts in tubes or 30 ml screw-capped bottles. Sterilize at 115°C for 10 minutes.

Identification: Purple pink solutions in tubes

Uses:

(1) It is an excellent stool specimen preservative and transport medium for faecal material. Prevents other intestinal organisms from overgrowing the enteric fever bacilli.

(3) *Cary and Blair's medium*

Disodium phosphate - 1.1 gms
Sodium chloride - 5 gms
Sodium thioglycollate - 1.5 gms
Agar - 5 gms
Distilled water - 991 ml

Add the ingredients to a chemically clean flask rinsed with Sorenson's 0.067 M buffer (pH 8.1). Heat with frequent agitation until the solution becomes clear. Cool to 50°C. Add 9 ml of freshly prepared aqueous 1% $CaCl_2$ and adjust the pH to 8.4. Distribute into previously rinsed and sterilized 9 ml screw-capped vials. Steam for 15 minutes Cool and tighten the caps.

Identification: Colourless semi-solid media in 9 ml screw-capped vials.

Uses: Used as a transport medium for V. cholerae

(4) *Venkataraman's-Ramakrishnan's media for V cholerae*

Boric acid - 0 3 gms
Potassium chloride - 0 3 gms
Distilled water - 100 ml

Adjust the pH to 9.2

Sterilize by autoclaving at 121°C for 15 minutes Dispense in 10 ml amounts in screw-capped bottles.

Identification. Watery solution distributed in 10 ml amounts in screw-capped bottles.

Uses. Used as a preservative medium to transfer stool suspected to contain *Vibrio cholerae*

2.5.9. Anaerobic Media

These media are used to grow anaerobic organisms.

Ex. 1 Robertson's cooked meat media (RCM)
2. Thioglycolate broth

(1) *Robertson's Cooked meat media (RCM)*

Preparation

(a) *Cooked meat*

Fresh bullock's heart - 500 gms
Water - 500 ml
NaOH 1N - 1 5 ml

Mince the heart and place in alkaline boiling water and simmer for 20 minutes to neutralize the lactic acid Drain off the liquid through a muslin filter and while still hot, press the minced meat in a cloth and partially dry it by spreading it on a filter paper.

(b) *500 ml of nutrient broth*

(c) *Preparation of complete medium*

Place meat in 1 oz bottles to a depth of about 2.5 cms and cover with 10 ml of nutrient broth. Autoclave at 121°C for 15 minutes

A tall column of meat is essential because conditions are anaerobic only where there are meat particles. There need be only sufficient broth to extend about 1 cm above the meat.

Identification:

Screw-capped bottles containing meat particles below and broth above.

Uses. Used in culturing anaerobic organisms like clostudium sp.

(2) *Thioglycolate broth*

Preparation

Yeast extract - 5 gms
Casein hydrolysate, pancreatic digest - 15 gms
Glucose - 5.5 gms
L Cystine - 0.5 gms
Agar - 0.75 gms
Sodium chloride - 2.5 gms
Resazurin sodium solution, 1 in 1000 - 1 ml
Water - 1 litre

Dissolve the ingredients other than thioglycollate and resazurin by steaming at 100°C. Add the thioglycollate and adjust the pH to 7.3 Add the resazurin solution, mix thoroughly, distribute in test-tubes or screw-capped bottles and sterilize at 121°C for 15 minutes Cool at once to room temperature (25°C) and store in the dark at room temperature. If more than the upper third is pink in colour, anaerobic conditions may be restored once only by steaming at 100°C for a few minutes

Identification

Brown solution in screw-capped bottles or tubes

Uses: Used for the growth of all anaerobic organisms

2.5.10. Media for the Cultivation of Fungi

Ex Sabaurand's glucose agar

Preparation

Glucose - 40 gms
Peptose - 10 gms
Agar - 20 gms
Water - 1 litre

Dissolve the ingredients in the steamer Filter through cotton gauze and adjust to pH 5.4. Autoclave at 115°C for 15 minutes Pour into petridishes or into tubes as slants

Identification:

Yellow coloured solid media in petridishes or slants in tubes

Uses:

(1) Used for the primary isolation of fungi from chemical material.
(2) Used for maintaining fungal stock cultures.

2.5.11. Media for Blood Culture

Both liquid media and biphasic media (Castaneda's method of blood culture) are used in the isolation of organisms from blood.

Ex: 1. Bile broth
2. Glucose broth
3. Biphasic media

(1) *Bile Broth*

Sterile nutrient broth - 1 litre
Sodium taurocholate - 5 gms
Sodium citrate - 2 gms

Mix the ingredients well Distribute it in 100 ml amounts in 4 oz blood culture bottles and sterilize by autoclaving at 121°C for 15 minutes.

Identification: Brown liquid media in blood culture bottles

Uses: Used in the selective cultivation of Salmonella typhi from blood.

(2) *Glucose Broth*

Nutrient broth - 1 litre
Glucose - 10 gms
Sodium citrate - 2 gms

Mix the ingredients well. Distribute in 100 ml amounts in 4 oz blood culture bottles. Sterilize by autoclaving at 121°C for 15 minutes.

Identification: Brown liquid in blood culture bottles

Uses: In the isolation of organisms from blood.

(3) *Biphasic media:* (Castaneda's method)

Eg: Brain heart infusion biphasic medium.

Preparation: Prepare brain heart infusion (BHI) agar according to the manufacturer's instructions.

Heat the ingredients in a steamer till the solution becomes clear. Distribute in 30 ml amounts in 1 oz 'medical flat' bottles (blood culture bottles) and sterilize at 121°C for 15 minutes. While hot, lay the bottles in a slanting position so that the medium is set as a slope extending upto the shoulder of the bottle. Pour about 20 ml of sterile BHI broth with sterile precautions into the BHI agar slants.

Identification:

Blood culture bottles containing both a solid slope and broth.

Uses: Used in the isolation of organisms from blood.

The advantages of the biphasic medium are:

(i) Number of subculturing is minimized, thus reducing the chances of contamination.
(ii) Organisms grow as columns directly on the slant.

2.6 Collection of Clinical Materials for Microbiological Investigations

Microbiological laboratories aid the clinician in many ways. First and foremost is the diagnosis of the disease process and the treatment of it. Hence, care should be taken during collection of the specimens at the bedside. The following general rules must be observed while collecting a clinical sample:

(1) The specimens should be from the suspected infective condition.
(2) Sterile swabs and sterile containers should be used to avoid contamination. These materials can be obtained from the microbiology department.
(3) The sample should be in sufficient quantity to permit thorough study.
(4) It is always better to send the sample before administration of an antibiotic. If this is not possible, the laboratory should be informed about the antibiotics used.
(5) The specimens should never be sent in antiseptics and disinfectants.
(6) The specimen should be clearly labelled with the patient's name, date of collection, time of collection and hospital number. This sample should be always accompanied by complete laboratory requisition forms with complete details like the patient's name, age, sex, nature of specimen, clinical features and its duration with probable clinical diagnosis.

2.6.1. Specimen for Bacteriological Investigations

(1) *Blood Culture*

The following apparatus is required: sterile 10 ml syringe, 21 gauge needle, antiseptic solution to clean the skin, spirit lamp.

Media for culture of different organisms.

Organisms	*Media*	*Quantity of blood*
Salmonella, cocci	50 ml bile broth 50 ml glucose broth	10 ml
Anaerobic organisms	Thioglycollate broth	5 ml
Brucella	Tryptose soya broth or Liver infusion broth	5 ml

General precautions while collecting blood. The skin should be cleaned properly with antiseptics — 2% tincture iodine and later with 70% alcohol The site should be allowed to dry and blood should be collected under aseptic conditions The blood should be collected in the following clinical conditions.

(1) Enteric fever
(2) Subacute bacterial endocarditis
(3) Brucellosis
(4) Any septicaemia

(a) For enteric fever, blood should be collected in the 1st week of the illness, for S.B E before the rise of temperature.
(b) For septicaemia, 4 frequent samples should be collected at subsequent temperature in the same day.

The samples of blood should be sent to the laboratory immediately.

(2) *Urine*

(a) Urine should be collected in sterile, wide-mouthed containers.
(b) In females· Midstream urine sample should be collected after cleaning the external genetalia with soap and water and further cleaning with spirit swab. In some cases, catheterization should be done, but catheterization is preferably to be avoided since there is a chance of introducing microorganisms into the bladder
(c) In males, the glans penis is cleaned with alcohol and midstream urine should be collected
(d) Urine specimens should be brought to the laboratory within one hour. If that is not possible, they should be kept in a refrigerator, but not for more than 24 hours

(3) *Stool samples·* These should be collected in sterile containers and immediately sent to the laboratory

(4) *Rectal swab:* This can be taken by introducing the sterile swab into the anus The swab should be replaced in a test-tube

(5) *Sputum culture.* The sputum should be collected in sterile screw-capped bottles

(6) *Throat swab culture:* This is a sterile swab fitted to a wooden stick kept inside a sterile test-tube with the outside fitted with a cotton plug. Make it point to always send two samples to the laboratory — one for smear-making and the other for culture The swab stick should be removed from the tube. Swab the back of the throat once, without touching the other parts of the mouth. Replace the swab in the tube. Dispatch it as soon as possible.

(7) *Nasal, nasopharyngeal and oral swabs* Nasal, nasopharyngeal and oral swabs should be collected in the same way as throat swabs.

(8) *Ear swab:* Two swabs should be taken, one for the mycological examination and the other for the bacterial cultures.

(9) *CSF cultures:* CSF should be collected under sterile conditions. 2-5 ml of it should be sent to the laboratory in sterile test-tubes or screw-capped bottle without any delay.

(10) *Conjunctival swab:* The swab should be taken before any local antibiotic therapy. If taken after, the suspected area should be washed with sterile saline and then the swab should be taken.

(11) *Cervical and vaginal swabs: Vaginal swab:* The site should be cleaned with Savlon after which the swab should be taken from the suspected site.

Cervical swab: The speculum should be used to visualize the cervix and a cervical swab should be taken under sterile conditions and sent to the laboratory.

(12) *Urethral swab and prostatic fluid·* Two samples should be sent to the laboratory, one for direct smear examination and the other for culture. The swabs should be sent to the laboratory immediately as they can be inoculated for Gonococci at the bedside.

(13) *Pus culture:* Pus from a closed abscess can be collected by a sterile syringe and needle, by aspiration, or a sterile swab can be used.

(14) *Serous fluid:* Under aseptic conditions, pleural, pericardial, ascetic, synovial bursal and hydrocele fluids should be collected in sterile test-tubes or screw-capped bottles; the citrated samples should be sent for cell count.

(15) *Biopsy and autopsy specimens* should be sent to the laboratory in sterile drop containers.

2.6.2. Specimens for Acid Fast Bacilli

(1) *Sputum:* Overnight sputum in a wide-mouthed sterile container should be sent to the laboratory.

(2) *Urine for tubercle bacilli:* In a clean bottle, the sample collected over 24 hours should be sent or the entire early morning sample should be sent.

(3) *Laryngeal swab and gastric lavage:* When very little sputum is present, as in the case of children who swallow sputum, the material should be obtained either by laryngeal swab or by gastric lavage.

Laryngeal swab: Two consecutive swabs should be taken from each patient.

Gastric lavage: Fasting stomach contents may be aspirated with a Ryle's tube in the morning. The sample should be sent within an hour of collection.

(4) *Pus from cold abscess:* A small quantity of pus can be sent in a sterile penicillin bottle.

(5) *Pleural and peritoneal fluids:* About 100 cc of fluid should be sent in a sterile flask to which citrate is added prior to the collection of fluids.

(6) *CSF:* About 5 cc of CSF (Cerebro-Spinal Fluid) can be sent in a sterile screw- capped bottle.

(7) *Faeces:* About 5 gms of faeces should be sent in a clean bottle.

(8) *Tissues:* Biopsy or autopsy specimens should be sent in sterile dry containers.

(9) *Tissue smear for Lepra bacilli:* Two smears should be sent for examination. Nasal swabs can also be sent.

2.6.3. Specimens for Anaerobic Culture

(1) *Pus:* Swabs should be taken from the deeper parts of the wounds and abscesses with a sterile stick swab. The stick should be broken at the tip and the swab should be kept in thioglycollate broth or RCM and sent to the laboratory immediately.

(2) *Blood:* About 5 ml of blood should be sent in thioglycollate broth.

(3) *Urine:* Midstream urine should be sent in thioglycollate broth.

(4) *Prostatic fluid:* Prostatic fluid should be collected under sterile conditions into the thioglycollate broth and sent to the laboratory.

(5) *Serous fluid.* Pleural fluid should be collected under aseptic conditions into the thioglycollate broth and sent to the laboratory.

2.6.4. Blood for Serological Tests

A dry, sterile syringe and sterile needle should be used The blood should be sent in sterile screw-capped bottles or sterile test-tubes. The sample must be accompanied with a requisition form containing all relevant information. Preferably, two samples of blood should be sent, one immediately after the admission and another a week later This is necessary to study the rise in antibody titre. For Widal, VDRL, Brucella agglutination test, RA latex fixation test, Paul Bunnell test, CRP test, and ASO test, send 5 ml of clotted blood in sterile test-tubes or screw-capped bottles.

2.6.5. Materials for Mycological Investigations

(1) *Skin scrapings:* The affected area is swabbed with a sterile gauze soaked in 70 per cent alcohol, and allowed to dry The lesion is scraped with a sterile surgical blade at the edge of the lesion These scrapings should be kept inside a sterile petridish or a sterile paper packet. This should always be accompanied with the requisite form furnishing available data.
(2) *Hairs:* The unhealthy hairs present at the site of a lesion should be plucked with sterile tweezers and kept in a sterile petridish and sent to the laboratory
(3) *Nails:* A piece of the affected nail and the scrapings from the under surface of nails are sent to the laboratory
(4) *Materials from systemic fungal infections.* Blood, spinal fluid, sputum, exudate from draining sinuses and abscesses, sternal marrow, scrapings from the edges of ulcers and abscesses and biopsy tissues are placed in sterile vials or petridishes or test-tubes and sent to the laboratory.
(5) *Swabs from mouth and vagina:* The swabs taken from the mucosal surfaces for moniliasis are placed in a sterile test-tube and sent to the laboratory
(6) *Biopsy specimens* These are sent in a sterile container for culture and animal inoculation.

2.6.6. Specimens for Parasitological Examination

(1) *Faeces:* For examination of parasites, ova and cysts freshly

voided faeces must be sent in a clean bottle

(2) *Blood for microfilaria* At least 2 cc of midnight sample of blood should be sent with the anticoagulant added to it

(3) *Other specimens* Materials like splenic puncture, liver puncture, pus from liver abscess, CSF, urine, rectal swabs and blood may also be sent for parasitological examination

NOTE: Stool for culture must be sent in a sterile bottle

2.6.7. Specimens for Virological Investigations

The diagnostic virology is entirely dependent on the correct selection of clinical specimens and their collection and transportation to the laboratory under appropriate conditions Specimens should be collected early during the course of the illness as successful isolation may be less in the later stages

All specimens should be collected in sterile containers Each specimen should be accompanied with a requisition form containing details about the patient like name, age, sex, ward and hospital number with a brief history of the nature of the specimen and the date of collection, and the examination required Most viruses are unstable at above 4°C, hence, specimens should be sent immediately, preferably kept chilled, packed in ice, to the laboratory.

For isolation of certain viruses, the specimens should be collected in a transport medium The common transport medium used for virological specimens is Hank's balanced salt solution + 10% bovine albumin + sodium carbonate + 50 units of penicillin + 50 mg of Streptomycin. The specimens that can be transported in this medium are swabs, biopsy and autopsy materials Serological tests can also be conducted where the isolation of viruses is impractical

Specimens for serological tests should not be frozen, but should reach the laboratory within 24 hours of collection

For serological procedures, two samples of clotted blood should be sent during the acute illness and another 14 days after the first sample. They should be sent in sterile, screw-capped bottles

(1) *Diseases of the Central Nervous System*

(a) *Faeces:* The specimen should be collected from the acute stage up to the second week, 5-10 gms of faeces should be collected in a sterile, screw-capped bottle The contents should be 1/3rd of the bottle. Freezing is not necessary, certain viruses are stable at normal room temperature.

(b) *CSF.* 5 ml should be collected during the acute stage of the illness The specimen should be chilled by keeping on ice cubes and sent to the laboratory

(c) *Throat swabs*· The specimen should be collected during the first few days of illness. After swabbing, it is immediately broken into the sterile screw-capped bottle containing 5 ml of transport medium

(d) *Neuropsy specimens:* Several grams of the brain substance can be sent to the laboratory in the frozen state.

(e) *Blood*· 5 ml of heparinized blood should be collected during the first few days of a suspected infection.

(f) *Saliva*· This is collected for suspected rabies cases

(g) Paired sera are collected for serological tests.

(2) *Disease of the skin:* Macules or papules should be scraped with a scalpel. Smears should be made on several glass slides Vesicles should be collected into a capillary tube These should be kept in screw-capped bottles. After removing the vasicular fluid, scrapings from the bases of vesicles should be taken and smeared on slides for microscopic examination. The smears must not be fixed with heat or by any other method The dried smears should be sent to the laboratory wrapped in clean paper

The specimens may be dangerous and proper care should be taken while handling them

(3) *Diseases of the eye:*Sterile swabs with a wooden stick should be used After collecting swabs from the diseased part of the eye, the tip of the swab should be cut and the swab should be kept in a screw-capped bottle containing 5 ml of transport medium At the same time, several smears can be prepared on glass slides

(4) *Diseases of the respiratory tract:* The specimens collected in the respiratory viral infections are mouth garglings, throat swabs and nasopharyngeal swabs. They should be collected in the first 3 days after the illness. After taking the specimens, they should be placed in screw-capped bottles containing 5 ml of transport medium and sent to the laboratory.

(5) *Materials for sterility tests:* Suture materials, gauze piece, cotton catgut, can be sent in sterile bottles or test-tubes Spore paper is supplied by the microbiology laboratory. This should be kept in the centre of the articles to be sterilized before autoclaving. The same should be sent to the laboratory to know whether the sterilization has been done properly or not Once in every 15 days, swabs may be collected from theatre equipment and sent to the laboratory for testing for anaerobic spore-bearing organisms.

PART - III

LABORATORY TECHNIQUES FOR DETECTION AND IDENTIFICATION

3.1 Methods for Laboratory Investigation

The detection of microorganisms and their identification in specimens sent for bacteriological studies are the chief concerns of the microbiology laboratory

The specimens may be of pus, urine, sputum, skin, C S F, nose or throat swab.

3.1.1. Problems and Procedure of Investigation

3 1.1 1 Problem of Contamination

Bacteria live in mixed populations everywhere This is a problem when you want to observe just one bacterial specimen not contaminated with other organisms. The contamination could come from the air, dust, desks, people, equipment, containers, glassware or other objects that may come in contact with the process of investigation

This problem of contamination is solved through the use of the *sterile technique*. Detection and determination of bacterial pathogens require a systematic study of bacterium in pure culture. The preliminary and basic study of a bacterium lies in the systematic determination of its cultural character (i e. gross morphology of the colony of the organism, morphology of the organism and its biochemical reactions)

3.1.1.2. Problem of Detection

The high-power lens of the microscope covers a volume of approximately one ten-thousandth of millilitre under one microscopic field Bacteria cannot be seen unless several (3 to 10) are present per high-power field, indicating concentrations of thirty thousand to one hundred thousand per millilitre No bacteria can be seen under the high-power if the bacterial specimen has a concentration less than

30,000 per millilitre. In such cases, the specimen is asked for culture. Specific bacteria are cultivated in specific media.

3.1.1.3. Laboratory Procedures for Investigation

The laboratory procedures employed to detect and identify microorganisms are·

(1) Microscopical examination
  (i) Direct examination of living microorganisms through the 'hanging drop method'.
  (ii) Examination of stained specimens
    (a) Gram stain
    (b) Ziehl Neelsen stain
(2) Culture
(3) Biochemical reactions
(4) Animal innoculation
(5) Serological tests
(6) Antibiotic sensitivity test

An outline of the above methods is given below

3.1.2. Microscopical Examinations

The smears of these specimens are subjected to microscopical examination to find out the presence or absence of the bacteria, and identify their morphological characteristics and staining reactions.

3.1.2.1. Direct Examination

The direct examination is most useful in obtaining an indication of the type of organism involved In some cases it also helps in establishing a diagnosis of the disease as in tuberculosis, leprosy, gonorrhoea.

It is also essential to identify and describe the various organisms in the specimen like leucocytes, red blood cells, epithelial cell, etc. An unstained preparation in bacteriology is mainly used for demonstration of bacterial motility and their reaction to a specific serum The best way to observe the motion of living bacteria is the *hanging drop method.*

3 1.2.2. Examination of stained specimen

Simple stains are used to show the presence of the organism and the nature of cellular contents in the exudates. A variety of staining methods are available. The most important of the stains employed in bacteriology are the Gram stain and Ziehl Neelsen stain The various groups of bacteria are identified from the stained preparation under the microscope They may be

(1) Gram positive cocci which may be arranged
  (a) in clusters (Staphylococci)
  (b) in chains (Streptococci)
  (c) in pairs
  (d) in fours, etc

They are found in pus, urine blood and other specimens

(2) Gram negative diplococci (rounded shape in pairs) which may be
  (a) shaped like coffee beans or
  (b) cluster in the cytoplasm of the leukocytes

They are found in urethral pus (gonococci) and CSF (meningococci) There are other Gram negative diplococci that are generally non-pathogenic They may be seen in a throat swab or sputum specimen

(3) Gram positive bacilli (Rod-shaped)
  (a) Gram positive Bacilli, with spores, Long and thick and may have square ends (anthrax) or rounded ends (tetanus, saprophytes) The spore appears as a large uncoloured area inside the bacillus and it does not stain with Gram stain
  (b) Gram positive bacilli without spores Usually small and variable in shapes, the ends may be swollen and arranged in rows or like letters. Found in throat specimen, blood, skin, etc (diphtheria, diphtheroids,, listeria).

(4) Gram negative bacilli Variable in size, ends rounded or pointed May be large and straight (colliform bacilli), comma-shaped (vibrio) or short and fat (proteus)

(5) Gram negative coccobacilli Quite variable in shape, not as rounded as cocci but not as long as normal bacilli (haemophilus). Found in a variety of body specimens.

(6) Spirochaetes· Irregular loose spiral, weakly staining Gram negative (treponema and barrilia)

(7) Acidfast tubercle bacilli.

(8) Non-acid fast bacteria

3 1 2.3 Reporting

In reporting the microscopical examination, a detailed description of all the elements and organisms found and their numbers are to be indicated

Elements Leucocyte, red blood cells, epithelial cells

Organism
Shape - cocci or bacilli, etc
Arrangement - pairs, chains, clusters
Staining properties
Gram positive or negative
Ziehl Neelsen acid-fast and nonacid-fast
Special characteristic - spores, granules, etc
Quantity - occasional, a few, a moderate number, many

Note It is rarely possible to diagnose a disease in the laboratory on the basis of the identification of the organism found by direct bacteriological examination of a specimen The result of such an examination, however, can help a physician to establish a diagnosis, when taken together with the symptoms shown by the patient.

3.1.3 Culture

A culture is the second essential step to determine the exact identity of bacteria It is particularly important to determine whether the organisms found in the specimen are pathogenic or non-pathogenic

The primary culture is to be carried out on solid culture media (agar in a petridish or test-tube) or in liquid media (broth in tubes)

Special factors must be taken into account when observing the cultural characteristics of an organism which are morphologically similar such as

(a) media which gives optimum growth,
(b) temperature,
(c) atmosphere,
(d) cultural characteristics, for example, size, shape and pigmentation.

When bacteria multiply on agar plates, they are called colonies They vary in size, shape, pigment, moderation and haemolysis on blood agar, depending on the type of media

Colonies are described as·
(a) smooth or rough,
(b) regular or irregular contours,
(c) mucoid, tenacious, dry or adhering to the medium,
(d) shiny or dull,
(e) discrete or swimming;
(f) (the nature of) pigmentation

3.1.4. Biochemical Reactions

Organisms which are similar in microscopic and cultural characteristics are differentiable by their reactions in biochemical tests

3.1.4.1 Fermenting of Sugar

Organisms when grown in sugar media have the property of fermenting some of the carbohydrates used and not others Glucose, lactose, sucrose, mannitol, maltose and ducitol are the sugars most commonly used

Each sugar medium has a coloured stopper and a set colour scheme may be established for the following sugars.

Glucose - Green
Lactose - Red
Maltose - Blue/White
Sucrose - Blue
Mannitol - Mauve
Sorbitol - Black/Blue
Ducitol - Pink

When an organism ferments the sugar in the medium, it produces acid and in certain groups, gas The production of acid is indicated by a change in colour of the medium, due to the inclusion of a pH indicator The production of gas is demonstrated by placing a small Durhan's tube upside down in the medium during its preparation (The tube must be completely filled with the medium before inoculating with the organism) If gas is produced, small bubbles of air will be seen inside the inverted tube

3.1.4.2 Other Biochemical Test

Organisms can be further identified biochemically by

(a) liquefaction of gelatin,
(b) production of indole,
(c) change in pH,
(d) urease production,
(e) production of hydrogen sulphides,
(f) Voges Proskaner reaction

3.1.5. Animal Inoculation

Animal inoculation is used (1) to detect specific diseases or tissue damage caused by viruses, and (2) to provide living culture media for microbes that are difficult or impossible to cultivate on artificial media. This method is useful for understanding clearly the nature, mechanism and cure of disease.

The animals most often used for this purpose are guinea pigs, mice and rabbits. Animals react to many kinds of bacteria as much the same way as man does.

3.1.6. Serological Tests

Serological tests are employed both for (1) identification of microorganisms; and for (2) the demonstration of antibodies in the serum of the patient. Refer to *Volume X* on *Serology* of this series for serological tests.

3.1.7. Antibiotic Sensitivity Test

This test is used to find out whether the organism is sensitive or resistant to a given antibiotic.

3.2 Hanging Drop Preparation

Aim

To study the motility, shape, relative size and arrangement of bacteria in a given broth culture.

Requirements

- Young broth culture
- Clean glass depression slide
- Clean coverslip
- Vaseline
- Spirit lamp/Bunsen burner
- Wire loop
- Compound microscope.

Procedure

- Take a clean coverslip and apply a little Vaseline to the four corners.
- Place a drop of broth culture in the centre of the coverslip, using a sterile wire loop.
- Invert a clean depression slide gently over the coverslip, the concavity facing the drop (i.e. downwards). See that the drop does not touch the slide.
- Quickly and carefully, turn the slide over, so that the coverslip is uppermost and the drop is suspended from the coverslip.
- Lower the substage condenser to reduce the light and use a concave mirror (excessive illumination renders the bacteria invisible).

- Partially close the iris diaphragm, if necessary (to secure sharp definition)
- Focus the drop under the low power objective so that the edge of the drop is exactly in the centre of the microscopic field
- Turn to the high power objective and focus, using fine adjustment
- Study the motility, shape, relative size and arrangement of the organisms
- Draw the diagram of the edge of the drop with the organisms and write your observations.

Comments

1 The term 'hanging drop' is self explanatory after the preparation i e the drop hangs from the coverslip.

2 The edge of the drop is focused because of the following reasons

(a) The contrast of (optic) aberration due to the two different refractive indices (of water and glass) is less at the edge of the drop

(b) The organisms move in a horizontal direction at the edge of the drop, contrary to the vertical movement seen at the centre of the drop

(c) Oxygen tension is more towards the edge of the drop and, hence, aerobes tend to accumulate at the edge of the drop.

Bacteria exhibits 'true motility' and 'false motility'. When a bacterium changes its position and moves in different directions, we consider it to be truly motile False motility is mainly due to Brownian movement i e the organisms exhibit an oscillatory movement without change of position due to the bombardment of the water molecule

3 True motility may be

(a) darting (missile-like), e.g *Vibrio cholerae*

(b) tumbling, e g *Salmonella*

(c) wriggling (worm-like), e g *Spirochaetes*

- *Spirochaetes*: Though lacking in flagella, move by the flexion and extension of their bodies
- *Bacilli* Motile bacilli *Salmonella*, *V cholerae*, *Pseudomonas*, *Proteus*, *Cl tetani*
- Non-motile bacilli *Klebsiella*, *Shigella*, *H influenza*, *Cl welchii*
- *Cocci* All cocci are non-motile except *Rhodococcus agilis*.
- *Anaerobes* Motility of anaerobes can be checked by inoculating them into a semi solid medium. Motile anaerobes are seen to spread at the deeper layers of the medium where the oxygen con-

tent is less or negligible

4. Diagnostic structural features:

- *Flagella*. These structures help in the motility of organisms They arise from the cytoplasm of the bacterium Bacteria may be Atrichate, Monotrichate, Amphitrichate, Laphotrichate or Peritrichate depending on the arrangement of the flagella. Flagella can be stained by Leifson's method, Loeffler's method and Fleming's method
- *Capsule*: The capsule is a gelatinous layer covering the bacterium and is immediately in contact with the cell wall (e.g. *Klebsiella*, *Proteus*)
- *Fimbriae*. Filamentous appendages arising from the cell wall They help in adhesion of Gram negative bacilli.
- *Spores*: Members of the genera *Bacillus* and *Clostridium* form highly resistant resting stages known as spores. They are produced to tide over unfavourable situations. Spores may be terminal (*Cl. tetani*), sub-terminal (*Cl welchii* and *Cl septicum*) and central (*Bacillus sp*). Spores can be stained by modified ZN method and Fuchsin nigresin method

3.3. Stained Preparation

Generally, bacteria are very tiny and colourless. One can observe bacteria under the microscope by making a hanging drop slide. One advantage of the stained bacteria smear slide over the hanging drop slide is that the bacteria are more visible Stained bacterial smears are also useful in helping to classify and identify bacteria. The most important of the stains employed in bacteriology are the Gram stain and the Ziehl Neelsen stain

The bacteria are readily discovered and studied when immobilized by fixation and stained in contrast to a bright background The staining on the immobilized bacteria imparts a distinctive colour to different kinds of bacteria.

3.3.1. Preparation of Smears

Preparation of smears for fixation of the sample in the slide is the prime requisite for examination of bacteria under a microscope. This is followed by staining.

Principle

The sample for examination (pus, sputum, urine after centrifuge, cerebrospinal fluid, etc.) are to be treated as follows.

- The sample is to be spread in a thin layer on a glass slide.
- It is to be dried completely
- It is then fixed on the slide by treating before the process of staining

Materials Required

- Inoculating loop.
- Clean grease-free slide which should be numbered like the specimen

- Bunsen burner.

Procedure

- Flame the loop until it is red hot by holding the loop vertically on the blue part of the flame.
- Cool the loop by waiting for 20 seconds.
- Take a portion of the specimen to be examined (for pus, by placing the loop flat on the surface of the liquid).
- Place the loop on the stick and press slightly and in the centre.
- By holding the loop flat against the slide, move the loop in a spiral manner from the centre in an outward direction Leave a space between the specimen and the margin of the slide
- Allow the slide to dry completely in the air.
- Flame the loop again as in step 1 to destroy any bacteria
- Check the smear to find if it is completely air dried and pass the slide through the flame of a Bunsen burner with the smear uppermost. Pass it through the flame three times. Allow to cool before staining.

Precaution

Unmarked smears are sometimes received in the laboratory from outside. In such cases, it is necessary to find out on which side the smear has been made For this, turn the slide so that it reflects the light from the window. The side without the smear will shine and the side with the smear will not reflect the light. It is always advisable to draw a circle around the smear with a grease pencil, so that it can be identified more easily.

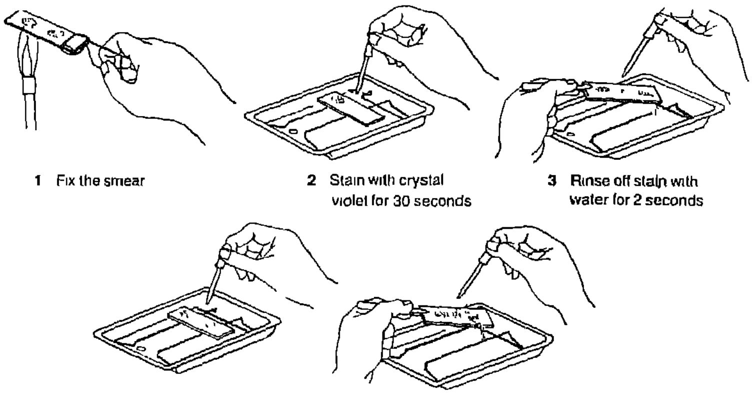
3.3.2. Gram Staining Process

In the Gram stain technique, bacterial cells are stained with one dye (an aniline dye of the violet series, usually crystal violet) and then flushed with an iodine solution. A decolourizing rinse (with 90% ethyl alcohol) follows. Finally, the smear is stained with a dye of contrast colour (safranin or carbol), so, in effect, two strains are used to differentially stain the bacterial cells.

Gram positive organisms retain the violet stain after decolourization with alcohol and are violet (purple) in colour In Gram negative organisms, the violet stain gets removed by alcohol and so they take on the colour of the counter stain, i.e., pink.

The Gram stain is done to classify bacteria into two groups:

(1) Gram positive : stained dark purple



1 Fix the smear

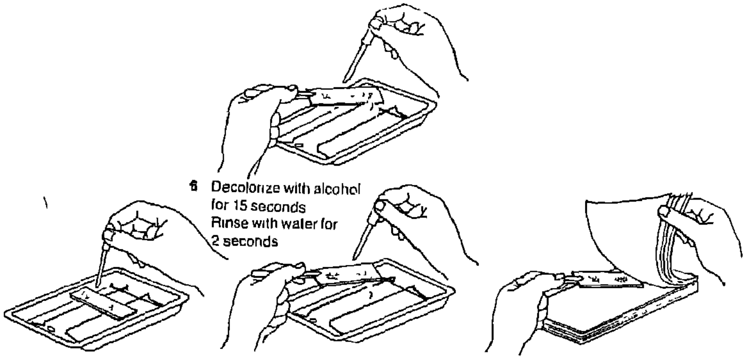
2 Stain with crystal violet for 30 seconds

3 Rinse off stain with water for 2 seconds

4 Cover with Gram's iodine stain for 30 seconds

5 Rinse with water for 2 seconds

FIG 3 3 1 (a) *Gram Staining*



6 Decolorize with alcohol for 15 seconds Rinse with water for 2 seconds

7 Stain with safranin for 30 seconds

8 Rinse with water for 2 seconds

9. Blot off water

FIG 3 3 1 (b) *Gram Staining*

(2) Gram negative · stained pink

Materials required

- Bacterial sample/culture
- Microscope slides
- Coverslips
- Staining tray or rack
- Inoculating loop

- Sterile water/tap water
- 1% Safranin stain
- 1% crystal violet stain
- Decolourizer of 90% ethyl alcohol
- Gram's iodine solution
- Bibulous paper booklet
- Medicine dropper
- Test tube holder
- Bunsen burner/spirit lamp
- Slide forceps
- Immersion oil
- Compound microscope

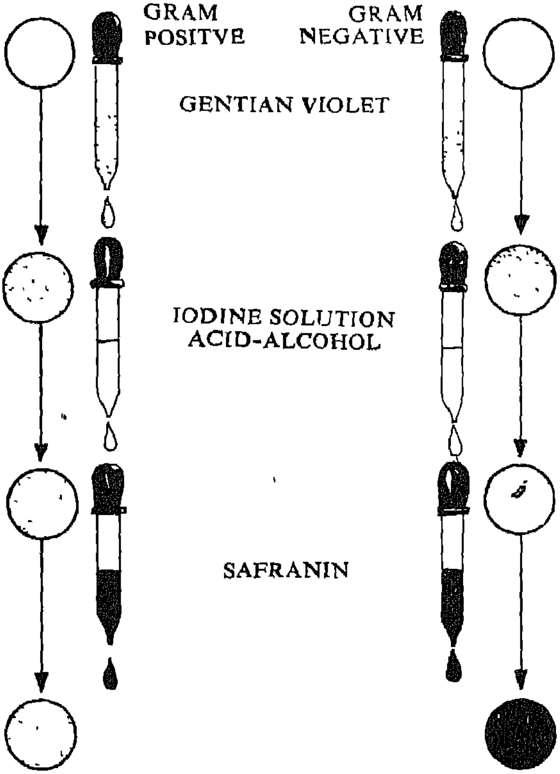


FIG 3.3.2 (a) *Gram Staining Process*

Procedure

- Take the slide with the fixed smear and place it on a staining rack/tray.
- Cover the bacterial smear with several drops of crystal violet stain for 30 seconds
- Rinse off stain with water for 2 seconds.
- Cover with Grams' iodine stain for 30 seconds.
- Rinse with water for 2 seconds.
- Decolourize with alcohol for 15 seconds.
- Rinse with water for 2 seconds.
- Look for patches, treat again with ethanol for 15 to 30 seconds, rinse well with water and drain.
- Stain with safranin for 40 seconds.
- Rinse with water for 2 seconds.
- Blot off water drops with bibulous paper; allow to dry in the air.
- Examine the Gram-stained slide under high power.

Observation

What is the advantage of Gram staining?

The bacteria can be identified or classified by their reaction to the stains.

In the Gram staining process, iodine fixes crystal violet to Gram positive cells so that the violet colour is not washed out by alcohol. Gram negative cells appear pink because alcohol washes out the violet colour before the cell is stained by safranin.

Gram +ve bacteria includes:

Bacillus
Clostridium
Corynebacterium
Lactobacillus
Micrococcus
Staphylococcus
Streptococcus

Gram-ve bacteria includes:

Brucella
Enterobacter
Escherichia
Haemophilus
Klebsiella
Neisseria
Proteus

Pseudomonas
Shigella
Salmonella
Vibrio cholerae

Importance

Gram's technique is one of the most important diagnostic tools in medical microbiology. Penicillin, for example, is much more effective against Gram positive bacteria. A doctor would be more likely to prescribe the pencillin antibiotic if it was known that the patient had an infection caused by Gram positive bacteria; and less likely to prescribe it if the patient had an infection caused by Gram negative bacteria. Streptomycin is more effective against Gram negative varieties.

Precautions

A false Gram negative reaction may occur when

1. the smear was fixed before it was dry;
2. the smear was too thick;
3. the Gram iodine solution was not thoroughly washed off;
4. the ethanol was not left along enough.
5. the safranin solution was too strong or left on the slide too long.

A false Gram negative reaction may occur when:

1. the Gram iodine solution was not left long enough;
2. the ethanol was left on too long and not washed off properly.

3.3.3. Ziehl Neelsen staining process

This staining technique is of great value in identifying bacteria.

Principles of Ziehl Neelsen Stain

The microorganisms are first stained a pinkish colour with a red dye called fuchsin. They are then immersed in a solution of acid (hydrochloric acid or sulfuric acid) and alcohol. The colour is "washed out". The bacteria are then counterstained with either a blue or green stain. Then the bacteria appear blue or green under the microscope. There are a number of bacteria that resist the washing-out action of acid—alcohol-fast bacteria. These bacteria retain the pink colouration and are seen under a microscope as red.

In this technique, acid-fast bacteria retain the carbol fuchsin stain; the acid-alcohol solution does not wash it out.

Non acid-fast bacteria are colourized and can be counterstained with other dye. Besides tubercle bacilli, acid-fast bacteria include also the bacteria causing leprosy

This test is mainly used for identification of tubercular bacilli found in sputum or body fluid

Quality of Specimen

The quality of the specimen is very important. For sputum, it is important to ensure that the specimen consists of purulent secretion coughed up from the bronchi and is not merely saliva from the mouth. Suitable specimens are best collected during a bout of coughing, after the patient wakes up in the morning.

Materials required

- Bacterial sample/culture
- Glass slides (preferably new and unscratched)
- Inoculating loop
- Cotton wool plug or metal wire for flaming
- Stop watch
- Staining rack
- Dropper

Reagents

- Carbon fuchsin for Ziehl-Neelsen stain
- Acid ethanol
- Aqueous methylene blue
- Methylated spirit
- Wash bottle with distilled water

Procedure

1. *Sterile transfer of bacterial specimen from the sample to the slide and preparation of a smear*

- Take two slides.
- Flame the inoculating loop until it is red hot, cool the loop by waiting for 20 seconds.
- Using the sterile loop, transfer a purulent portion of the specimen (5 PUTNM) to each slide
- Make the smear by tracing concentric circles, well separated, leaving a margin at the edge of the slide.
- Reflame the loop to sterilize.

2. *Fixation of the smear*

- Air dry the smear

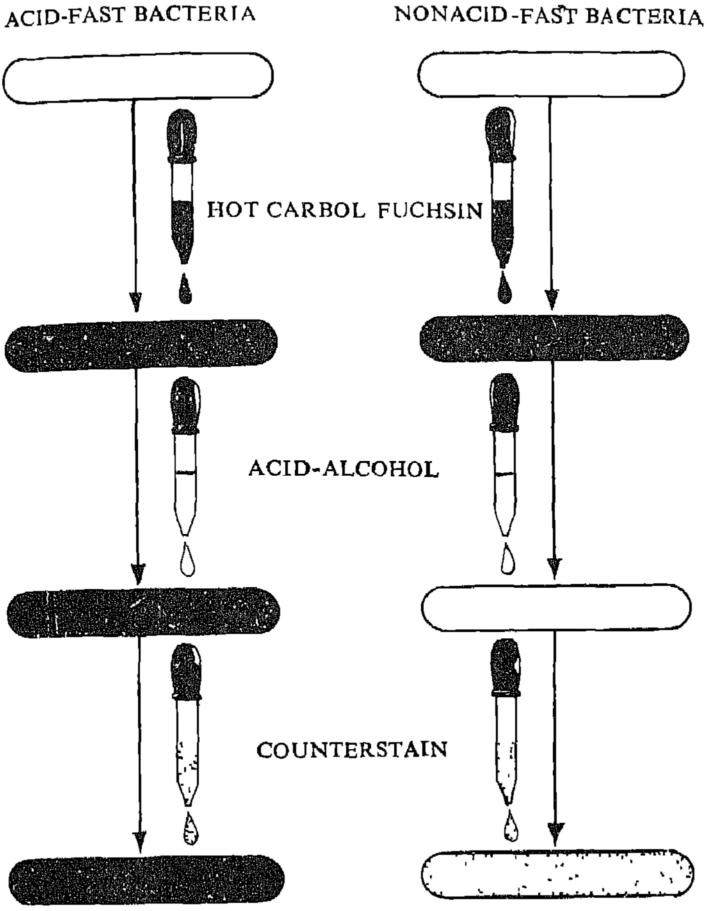


FIG 3.3 2 (b) *Acid-fast Technique*

- Fix the bacteria by passing the slides three times through the flame.

3. *Procedure of Ziehl-Neelsen staining*

- Place the slide on two glass rods over the sink
- Pour carbol fuchsin over the slide.
- Drop the cotton wool swab in methylated spirit, ignite and pass slowly under the slide to heat.
- Once the steam rises, set the stop-watch for 5 minutes and continue heating the slide without allowing the liquid to boil If the

filtered stain starts drying, add more carbol fuchsin to avoid drying.

- Allow the slide to cool.
- Wash the slide gently with water until the water that runs off is colourless.
- Pour acid ethanol on the slide and leave it for 2 minutes
- Wash the slides in ordinary tap water and drain.

Note: Examine the slides to see if they are completely decolourized or not. If there is any trace of fuchsin apply acid ethanol again and leave for 1 minute.

- Wash the slide with water and check if the slides are completely decolourized.
- Pour methylene blue over the slide and leave it for 30 seconds.
- Wash with tap water for 1 minute and drain off the water.
- Allow the slides to dry by putting the slide on a slide rack.

4. *Examination of the slide under oil immersion objective*

- Place the slide under the oil immersion objective.
- Observe the bacteria.
- See the bright red rods on a blue background. The rods are straight or slightly curved, often granular, arranged in groups of 3-10 bacilli close together. These are tubercle bacilli.

Precautions

On examination, precautions are to be taken to avoid the mistake of identifying the following as tubercle bacilli:

- Yeast is stained more or less red When heated they often break up into large red groups.
- Spots of stain deposit (when the slide is not properly decolourized.)

Modified Ziehl Neelsen stain

This is used for leprosy which is less acid fast than the tubercle bacilli. The procedure is exactly the same except that acid ethanol is modified (to contain 1 ml concentrated hydrochloric acid, 66 ml 95% ethanol and 33 ml distilled water). The methylene blue used above is also modified to contain 30 ml 95% ethanol and 70 ml distilled water. All the steps are the same as those for the tubercle bacilli.

In the case of diagnosis of leprosy, the smear is taken from a union (if required by incision on the site, superficially). Serous material from the incision is spread on a slide, air dried for 3 minutes in formaldehyde and examined under a microscope after the modified Ziehl-Neelsen method.

3.4 Making a Sterile Transfer

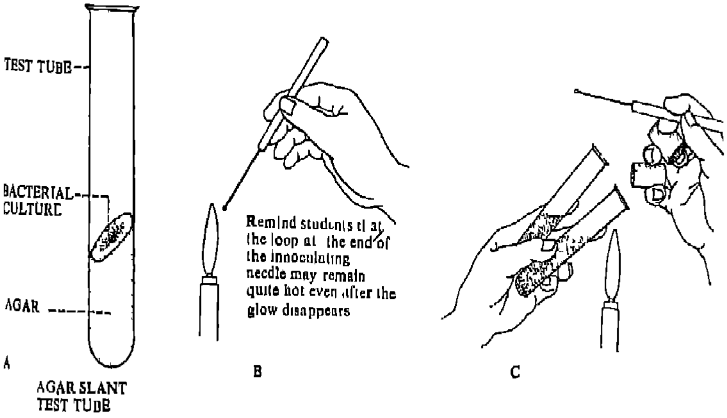
There are various possible sources of contamination in culture transfer. The contaminants could come from the air, dust, desks, people or any other objects that may come in contact with the process of transfer. So, the process of transfer should be made sterile

Materials required

- A test-tube with broth culture of *Vibrio cholerae*
- A test-tube with fresh broth medium.
- Bunsen burner/spirit lamp
- Inoculating loop
- Labels
- Disinfecting solution
- Antiseptic (hand soap)

Procedure

- Clean the surface of your work area with a disinfectant.



Remind students that the loop at the end of the innoculating needle may remain quite hot even after the glow disappears

FIG 3 4 1 (a) *Making a Sterile Bacterial Transfer*

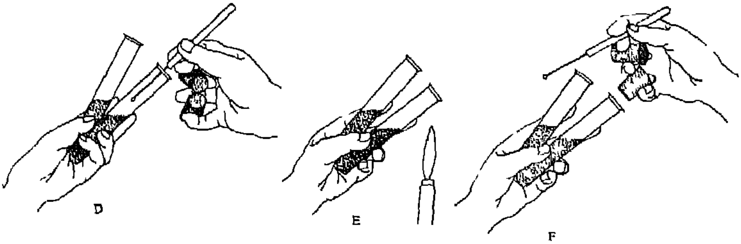


FIG 3 4 1 (b) *Making a Sterile Bacterial Transfer*

- Pour the disinfectant on the table top; spread it thinly around with a paper towel
- Allow the thin film to air dry In research laboratories, entire rooms are disinfected in this way to get rid of possible contaminants
- Scrub your hands with an antiseptic soap Rinse your hands
- Hold the inoculating needle in the flame of a Bunsen burner.
- The needle becomes sterilized after it turns red hot
- Use your thumb to hold both test-tubes (one with V cholerae culture and the other with fresh agar)
- Remove the plugs from the tubes with your free hand by grasping the plugs between your fingers.
  Note: Never place the plugs on the table top or on any other surface Doing this might contaminate the plugs
- Sterilize the open ends of both tubes by placing the open ends of the tubes in the flame for two seconds.
- Insert the inoculating needle in the culture tube, and gently touch the tip of the needle on the surface of the broth of the culture tube.
- Slowly withdraw the needle
- Transfer an overall amount of the culture by carefully and gently moving the needle across the fresh broth surface
  Note. Do not touch the needle to the sides of the tube as you withdraw the needle.
- Reflame the mouths of the test-tubes to sterilize them.
- Replace the plugs
- Reflame the inoculating needle to sterilize it.
- Label the test-tube you inoculated with your name, date and the name of the culture
- Incubate the inoculated culture
  Note This incubated culture is used for laboratory study

3.5 Different Methods for Isolation of Bacteria

In order to study the cultural characteristics of a bacterium, it is essential to obtain it in pure culture uncontaminated by other bacteria

Culture are usually prepared on solid or liquid media in petridishes by streaking with a platinum or nichrome wire loop The loop is sterilized in a flame and when cool, is charged with material It is important that the surface of the medium should be dry, otherwise separate colonies may not be obtained since the growth will be confluent

Inoculation of plates

(a) by streak method
(b) by pour plate method
(c) by spreading or carpeting

3.5.1. Streak Plate Method

This can be carried out
(1) by successive/consecutive rubbing
(2) by grid plate method
(3) by heating and subsequent streaking

3.5 1.1(a) By Successive Rubbing

The surface of the solid medium on the plate is streaked with the wire loop charged with the inoculum. At first, the primary inoculum is made and then parallel streaks are drawn without intermittent heating of the loop, touching the previous streaks with the loop

3 5.1.1.(b) By Consecutive Rubbing

It is the same as above. The parallel streaks do not touch each other

3 5.1.2. By Grid Method

This is usually employed for inoculation of highly selective media like DCA, S S. Agar, etc Here the streaking is done by a set of parallel streaks over the entire surface of the plate, set initially at 90° and then at 45°.

3.5.1.3 By Heating and Subsequent Streaking

This method is employed to inoculate materials which are flooded with a greater number of organisms along with commensals e.g stool, throat swabs, etc The primary inoculum is made and the loop is sterilized and charged again over the primary inoculus and streaking is carried on. The same procedure of sterilization and recharging the loop from the previous streaks is followed

3.5.2. Pour Plate Method

This procedure entails mixing a bacterial culture and the agar medium cooled at 45°C and then pouring into a plate, allowing it to set.

3.5.3. Carpeting or Spreading

This method is used to grow the organisms uniformly on the surface of the plate This is done with the help of a spreader.

3.5.4. Cough Plate Method

The medium (e.g.: Bordet Gengou's medium) is exposed for direct inoculation as the patient coughs in front of the plate held at a distance of 15 cm

3.5.5. Nasal and Slit Smear for Lepra Bacilli

3.5.5.1. Slit Smear

Slit smears are made from lesions and standard sites such as the ear lobes, chin and forearms. A superficial incision is made in the dermis with a sharp scalpel, care being taken to avoid bleeding. The tissue fluid is then scraped out with the tip of the blade held at 90 degrees to the direction of the incision and a smear is made on a microscope slide. After staining with Ziehl- Neelsen technique, the slides are examined using the oil immeision lens (x 100 magnification objective) and the bacterial index (BI) is assessed as follows.

BI 6+ : Numerous clumps of bacilli in every field
BI 5+ : 100 to 1000 bacilli/field
BI 4+ : 10 to 100 bacilli/field

BI 3+ : 1 to 10 bacilli/field
BI 2+ : 1 to 10 bacilli in 10 fields
BI 1+ : 1 to 10 bacilli in 100 fields

The Morphological Index (MI)

This is the percentage of solid staining organisms. It is generally assumed that organisms which stain in a poor or fragmented fashion are dead although this may not be absolutely true. This examination gives a good index of bacterial viability. Consequently, the MI enables the effect of chemotherapy to be monitored and the emergence of drug resistance to be detected.

3.5.5.2. Nasal smears

Smears from nose blows or scrapings from nasal septum are made on a glass slide and stained for leprosy bacilli by the Ziehl- Neelsen method and observed under the oil immersion lens.

3.6 Culture of Various Clinical Specimens in the Laboratory

3.6.1. Urine

Urine is asked for culture in urinary tract infections

Direct Examination

Wet film· For the presence of pus cells and erythrocytes and casts.

Grams stain To know whether bacteria are present in urine or not preliminarily

Culture,

Depending on bacterial count in the Gram smear, the urine is diluted with sterile normal saline. If there are no bacteria in the Gram smear, there is no need of diluting the urine. 0.01 ml of urine is taken with a measured loop (diameter of the loop is 4 mm) and cultured on McConkey agar and blood agar. incubated at 37°C for 24 hours At the end of 24 hours, the colonies are counted.

(1) If the colony count is more than 1,00,000 colonies per ml, this is termed as *significant bacteriurer*.

(2) If the bacterial count is between 10,000 and 1,00,000 per ml, *significance is doubtful*; further specimens should be obtained.

(3) If the colony count is less than 10,000 bacteria per ml, it is regarded as *contaminant* (unless the patient is on treatment for a known urinary tract infection).

The colony morphology is studied by the smear and identified by further biochemical tests. The antibiotic sensitivity test is also done to know the sensitivity pattern of the organism

3.6.2. Throat Swab

Depending on the clinical condition, the throat swab can be processed as follows·

Either it can be sent for sore throat which is caused by B haemolyte streptococci or diphtheria caused by *Corynebacterium diphtheria*

Dimet Smears. Take a throat swab and make two smears

(1) Stain one smear with Gram's stain

(2) Stain second smear with Alberts stain

(a) If only Gram positive cocci in chains are seen in the Gram's smear the throat swab should be cultured on blood agar and incubated at 37°C for 24 hours At the end of the 24 hours, blood agar, the colony morphology and haemolysis are studied Sensitivity test is also done.

(b) If the Gram stain shows Gram +ve bacilli and Alberts stain shows greenish pigmented bacterial body and bluish-black metachromatic granules, diphtheria is suspected. Presumptive diagnosis is given to the clinician and the following media are used·

(1) Loffler serum

(2) Potassium tellarite blood agar incubated at 37° for 24 hours After 24 hours, the colony morphology is studied by smear and further identified by biochemical tests Confirmative diagnosis of diphtheria is given to the clinician after doing the toxigenicity test *in vitro*

3.6.3. Sputum

For the following clinical conditions, sputum is asked for culture routine lower respiratory tract infections like pneumonia, bronchitis, occasionally, tuberculosis, and rarely, any fungal infections

(1) *Gram film*: Make a smear from sputum and observe carefully the presence of predominant organisms, a provisional diagnosis may be given (e.g · pneumococcal infection).

(2) *Ziehl-Neelsen film*. Examine for acid and alcohol fast bacilli, if present, a presumptive diagnosis of tuberculosis can be made.

Culture

(a) Prevalent portion of the sputum is cultured in blood agar, McConkey agar and chocolate agar incubated at 37°C for 24 hours. The colony morphology is studied by smear and further identified by biochemical tests and the antibiotic sensitivity test is also done

(b) For tuberculosis, the sputum is homogenized with an equal volume of 4% sodium hydroxide It is then centrifuged at 3000 r p m for 30 minutes. The sediment is neutralized with 2/10 HCl The smear is made from this concentrated material. Stained by the Ziehl Neelsen method, the material is inoculated on (Lower stains) Lomensteins Tensens medium, incubated at 37°C over a period of 6 weeks. Every week, the media should be checked for the evidence of growth.

(c) For fungal infections, the prevalent part of the sputum is inoculated on one pair of Sabourand's glucose agar or is incubated at room temperature, and another part is incubated at 37°C. The media should be kept for a minimum of 3 weeks before it is discarded

3.6.4. Blood Culture

Blood culture can be asked for by clinicians for Enteritis pyelitis of unknown origin, subacute bacterial endocarditis and septicaemias.

* Culture bottles inoculated with 5 ml of blood are:

Bile broth
Glucose broth
Liver infusion broth

The broths are incubated at 37° for 24 hours. After 24 hours they are subcultured on blood agar and McConkey agar and the media are incubated at 37°C for 24 hours. After 24 hours, the plates are observed for any growth. If there is no growth, the sub culture should be made again on the 3rd day. So, every alternate day sub culture should be done till the 7th day for the bile broth and glucose broth cultures before discarding as negative. Subcultures in liver infusion broth should be continued for every 3rd day for two weeks before discarding as negative.

If there is any growth on plates, the colonies (*Salmonella*) are identified by the slide agglutination test with polyvalent and monovalent antisera, and further identified by biochemical reactions. Antibiotic sensitivity should be put up to know the sensitivity pattern of the organism.

3.6.5. Stool Specimen

Faeces culture can be asked for in gastro culturitis, and bacillary dysentery and diarrhoeas. According to the clinical condition, the culture should be done.

Usually, stool culture is done in the following way:

Faeces is indulated on blood agar, McConkey agar and Selanite F broth, incubated at 37°C for 24 hours. After 24 hours, the colonies are observed and identified by biochemical reactions. Antibiotic sensitivity can be put up. From Selanite F broth, subculture is done on McConkey media These colonies are also identified by biochemical reactions and antibiotic sensitivity is put up, which will aid in the treatment of the case.

3.6.6. C.S.F.

The specimen CSF is asked usually for in dealing with meningeal symptoms and any disorder of the central nervous system caused by microorganisms.

Processing: The CSF is centrifuged at 3000 r.p.m and the deposit is taken. The deposit is used for Gram staining as well as culture If the Gram stain *reveals any bacteria* the report should be sent as *presumptive diagnosis of bacterial* infection. The material is further cultured on blood agar, chocolate agar and McConkey agar. Blood agar and chocolate agar are incubated at 37°C into 5% CO_2 tension for 24 hours After 24 hours, smears should be made for study, antibiotic sensitivity should be set up, and the organisms should be further identified by biochemical reactions.

3.6.7. Pus

Usually, pus should be sent from cases of wound infections, abscesses in sterile tube.

Culture

Smear: Gram's stain can be done on a smear prepared from pus

Culture: It should be cultured on Blood agar and McConkey agar; part of the pus is inoculated into the Robertson's cooked meat medium for anaerobic microorganisms incubated at 37°C for 24 hours. After 24 hours, the colonies are noted. The morphology is studied by smear and antibiotic sensitivity is put up The colonies are identified by biochemical reaction The pus inoculated into the R.C.M. is processed anaerobically.

3.6.8 Common Culture Media

The common culture media that can be used in a microbiological laboratory are as follows:

3.6.8 1 Liquid Media

(1) Nutrient Broth	:	Basal media used for preparation of nutrient agar.
(2) Peptone water	:	Basal media used for preparation of sugar sets used for study of the biochemical characteristics of microorganisms.
(3) Glucose Broth	:	Used for isolation of pyogenic cocci.
(4) Bile Broth	:	Used for isolation of *Salmonella* group

		of organisms.
(5) Brain heart infusion Broth	:	Used for isolation of both cocci and Gram negative bacilli.
(6) Liver infusion Broth	:	Used for isolation of *Brucella* organisms
(7) Thioglycollate Broth	:	It is an anaerobic medium used for isolation of anaerobes like *Clostridia* and bacteroids.
(8) Selanite F Broth	:	Used for isolation of *Salmonella* and *Shigella* from faeces specimens.
(9) Alkaline Peptone water	:	Used for cultivation of *Vivrio cholerae*
(10) Tetrathionate Broth	:	Used for cultivation of enteric group of organisms.
(11) Sugar media	:	Used for study of biochemical reactions of microorganisms.

3 6.8 2 Solid Media

(1) Nutrient agar	:	(1) Simple medium, wide range of non-fastidius microorganisms will grow in this media. (2) Used for production of pigment in some microorganisms (3) Used for antibiotic sensitivity testing.
(2) Blood Agar	·	It is an enrichment media used for cultivation of the following organisms (1) β haemolytic streptococci (2) α haemolytic streptococci (3) Non-haemolytic streptococci (4) *Bordetella pentussis* (5) *Haemophillus influenza* (6) *Staphylococcus aureus*
(3) Chocolate Agar	:	Used for cultivation of (1) Pneumococci (2) Gonococci (3) Meningococci
(4) McConkey's Agar	:	(1) Used for cultivation of Gram negative bacilli (2) It differentiates between lactose fermenting and non-lactose ferment-

	ing Gram negative bacilli
(5) Wilson & Blair's Media	Used for isolation of *Salmonella* group of organisms.
(6) T G.B S Media	Used for isolation of *V cholerae*
(7) Bandet-Gauge Agar	Used for isolation of *Bordetella pertusis*
(8) Lowenstein Jewsens Media	Used for isolation of (1) *Mycobacterium tuberculosis* (2) *Mycobacterium bovis* (3) *Atypical mycobacteria*
(9) Brain heart infusion Agar	Used for isolation of Both pyogenic cocci and Gram -ve bacilli
(10) Tellurite Blood Agar	Used for cultivation of: (1) *Corynebacterium diptheriae* (2) diphtheroids
(11) Loffler's Serum	Used for cultivation of *Corybebacterium diphtheriae*
(12) Labourand's Glucose Agar	Used for cultivation of many of the fungi
(13) Robertson's cooked meat media	It is an anaerobic medium used for cultivation of anaerobes like clostridia and bacteroides.
(14) Deoxycholate citrate Agar	Used for cultivation of *Salmonella* and *Shigella*

3.7. Anaerobic Cultivation

3.7.1. Aerobes and Anaerobes

Depending on their oxygen requirement, microorganisms can be classified into 4 groups:

(1) *Obligate Aerobes*:	These microorganisms which require free oxygen for their growth. *Ex:* (a) *Alkaligenes faecalis* (b) *Meraxells* (c) *Brucella.*
(2) *Obligate Anaerobes*:	These organisms that can live and grow only in the absence of oxygen, i.e. under high reducing intensity and low redox potentials. *Ex:* (a) *Trepenema denticola.* (b) *Clostridium tentani.* (c) *Clostridium betulinum.*
(3) *Facultative Anaerobes*	Those organisms which can live and grow in the presence as well absence of oxygen Most of the bacteria come under this group *Ex*: (a) Staphylococci, (b) *E.coli*, etc.
(4) *Microaerophilic Organisms*:	These organisms that grow in presence of just traces of oxygen. *Ex*: (a) *Vibrio sputerum.* (b) *Clostridium welchii*, etc.

3.7.2. Source of Anaerobes

Anaerobes occur in a variety of human body sites They are usually present as normal commensals without causing any harmful effects.

But under predisposing factors, i.e whenever there is low redox potential, they can act as pathogens, thus, producing endogenous infections. During infection, they are isolated from various specimens like faeces, blood, pus, exudates, etc

3.7.3. Methods of Creating Anaerobiosis

(1) By using certain gases like, (a) 100% H_2.
(b) 90% H_2 + 10% CO_2.
(c) 80% H_2 + 10% N_2 + 10% CO_2.
(2) Addition of reducing agents to the media like,
(a) Thioglycollate.
(b) 10% Ascorbic acid.
(c) Meat particles as in RCM.
(d) Palladium chloride.
(e) Cysteine, etc.,
(3) Growing anaerobes deep in agar butts.
(4) Simultaneous growth of aerobe and anaerobe.
(5) Biological method by employing germinating seeds.
(6) A simple method, devised by Buchner, employs a filter paper having the same diameter as a petridish. This filter paper is placed on the top of one half of the petridish and a mixture of pyrogallic acid and sodium hydroxide in dry powder form are spread on it. Then, the other half of the petridish containing the media with the inoculated specimen is inverted over the filter paper and the edge is sealed tight with molten wax. The alkaline- pyrogallate mixture will absorb all the O_2 present within the closed system, thus creating complete anaerobiasis.
(7) Utilization of anaerobic jars.

3.7.4. Anaerobic Cultivation in Anaerobic Jar

MoIntosh and Filde's Anaerobic Jar

This jar is used to provide a complete anaerobic environment for the cultivation of anaerobic bacteria like *Clostridia*, etc. It is a stout metallic jar with a metal lid Inoculated culture plates are placed inside the jar and the lid is clamped tight. The lid has 2 tubes: (1) outlet tube, which is connected to a vacuum pump to evacuate the air inside, (2) gas inlet, which is then connected to a hydrogen supply. The lid also has 2 electric terminals, leading from which and suspended on the underside of the lid, is a small porcelain spool, around which is wrapped a layer of a palladinized asbestos. After filling the jar with H_2, the electric terminals are connected to a current supply to heat the palladinized asbestos This acts as a catalyst for the combination of H_2 with residual O_2 present in the jar to form water An indicator, i e. reduced methylene blue is employed for verifying the anaerobic condition in the jar, which remains colourless anaerobically but turns blue on exposure to O_2 The jar with the complete anaerobiosis is incubated at 37°C for 48 hours and the growth of anaerobic organisms is noted

3.8 Water Analysis

3.8.1. Collections of specimens

The specimens are collected in bottles of 230 ml (or 6 oz. screw-capped bottles) capacity with ground- glass stoppers.

The bottles are sterilized by autoclaving. The neck and stoppers are covered with craft paper which is removed only while collecting the specimen.

Care should be taken during collection to avoid any bacterial contamination from an outside source.

If the specimen is taken more than 3 hours before analysis, it should be kept in ice.

If the sample is taken from a chlorinated water source, a crystal of sodium thiosulphate is introduced into the sampling bottle to effect neutralization.

The routine tests generally used in bacteriological examination of water are:

(1) A quantitative test for all coliform bacilli known as "Presumptive Coliform Count".

(2) A differential test for typical coliform bacilli (*E. coli*) known as the differential coliform test or Eijkman test.

3.8.2. Presumptive Coliform Count

An estimation of the number of coliform bacilli in a water supply is usually made by adding varying quantities of water to measured amounts of single and double strength modified McConkey's broth sterilized in bottles with Durham's tubes The size of the bottle varies with the quantity of medium and water to be added to it. The Durham's tube indicates gas production.

With sterile graduated pipettes, the following amounts of water (given in the table) are added. This can be recorded in a tabular form which will help to record the production of acid or acid plus gas.

Media with water sample	Growth	
	acid ⊥	acid + gas +
50 ml DSM + 50 ml Water		
10 ml DSM + 10 ml Water		
5 ml SSM + 1 ml Water		
5 ml SSM + 0.1 ml Water		

DSM = Double Strength McConkey's medium
SSM = Single Strength McConkey's medium

The bottles are incubated at 37°C and after 24 hours, the reading is taken. The number of bottles showing the production of acid and gas is recorded on the above table. Those that show acid and gas are considered to be "presumptive positive" as a result of the growth of coliform bacilli. In reporting the results of the presumptive test, reference is made to the McCardy probability table. With this table and the various combinations of positive results obtained, the probable number of coliform bacilli in 100 ml of water can be read.

The result is read as probable coliform count. .... organism/100 ml of water.

3.8.3. Differential Coliform Test (Eijkman Test)

This is done to confirm whether coliform bacilli detected in the presumptive test are *E.coli* (*Escharitia coli*). This depends on the ability of *E.coli* to produce gas when grown in McConkey's broth at 44°C and inability of a typical coliform bacilli to do this.

Method: Subcultures are made from all the bottles showing acid and gas in the presumptive coliform test (in single strength McConkey's medium) in test-tubes containing Durham's tube. Indole paper is introduced to see whether indole is produced. These tubes are then incubated at 44°C in a water bath for 24 hours. Those yielding gas and producing indole are regarded as containing *E.coli*. Again, with the help of McCardy's table, the number of *E.coli* in 100 ml of water can be estimated. This is the confirmed *E.coli* count.

It is stressed that even a single *E.coli* in 100 ml of water should be viewed with suspicion and proper treatment of the drinking water source is advisable.

3.9. Bacteriological Examination of Milk

This is done for hygienic conditions for large amounts of milk supplies and milk distribution in dairies.

This consists of:

(1) an enumeration if viable bacteria presence in a given quantity,
(2) a quantitative estimation of contamination by coliform bacilli;
(3) the determination of the presence of specific pathogenic organisms. (Eg. *Mycobacterium tuberculosis*)

3.9.1. Sampling

(a) If the milk is in retail bottles or packets, one unopened bottle or packet is delivered to the laboratory.
(b) When in a bulk container, the milk is mixed carefully by a sterile plunger, and then a sample is collected in a sterile 150 ml (4 oz.) screw-capped bottle.

Samples should be dispatched immediately to the testing laboratory.

3.9.2. Technique of estimating the number of viable bacteria.

This is done by the "plate count" method. The media used is yeast extract milk agar. A series of dilutions of the milk sample is made in sterile bottles with sterile tap water. For each dilution, a separate sterile pipette should be used. The following dilutions are used.

Water Milk

1 in 10 — 90 ml + 10 ml
1 in 100 — 90 ml + 10 ml of 1 in 10 dilution
1 in 1000 — 90 ml + 10 ml of 1 in 100 dilution

One ml of the diluted milk is placed with a sterile pipette in a sterile petridish and 10 ml of melted agar cooled to 45°C is added and mixed with the milk by rotating the plate carefully so that organisms, if any, are uniformly distributed throughout the agar. After the medium has solidified, the plates are incubated in the inverted position for 72 hours at 37°C.

The number of colonies including pinpoint colonies is counted in each plate and the mean is calculated This multiplied by the dilution is reported as the number of viable bacteria/ml.

Under most favourable conditions, a specimen of raw milk may contain at least 500 bac./ml but under bad conditions it may reach even several millions/ml.

3.9.3. Test for Coliform Bacilli

Varying amounts of milk are added to tubes or both containing McConkey's fluid medium. Milk can also be diluted from 1 in 10 to 1 in 10,000 and tested in screw-capped bottles

Three 10 ml of the above medium is inoculated with 1 ml of the diluted milk and incubated at 37°C for 48 hours. The tubes are examined for acid and gas production and the milk is said to be good if acid and gas are absent in 2 out of 3 bottles.

3.9.4. Test for Tubercle Bacilli

The sample is thoroughly mixed and a quantity of 100 ml is divided into 50 ml amounts and centrifuged for 30 minutes at 3000 rpm. The sediment in each tube is suspended in 2.5 ml of sterile saline solution. With this.

(a) A smear is made and stained by the Z.N method of AFB

(b) Two guinea-pigs are injected s.c. on the inner side of one thigh with the suspended sediment and kept under observation. One guinea-pig is killed at the end of four weeks and an autopsy carried out. If it shows no tuberculous lesions, the other guinea-pig is killed at the end of the 8th week.

3.9.5. Other Tests

Other tests done for detection of micro-organisms in milk are as follows.

3.9 5.1. Methylene blue reduction test

This test has been used since 1936 as a substitute for bacterial count.

It depends on the reduction and decolourisation of the dye methylene blue by the bacteria in milk. The rate of reduction helps to measure the degree of bacterial contamination.

3.9.5.2. Phosphatase test

This is done for pasteurized milk. This test determines inactivation of the enzyme phosphatase normally present in cow's milk.

The standard method for detecting the presence of the enzyme is based on its ability to liberate p-nitrophenol from disodium p-nitrophenyl phosphate. This yields yellow colouration and the result is read with the help of the Lovibond comparator.

3.9.5.3 Test for Brucella Infection in Milk

In infected cows, the brucella organisms tend to localize in the udders and are excreted in the milk. The most common test done for the detection of this is the milk ring test The milk of cows suffering from brucella infection of the mammary gland may contain brucella agglutinins, as said above. On the addition of a conc. suspension of *B. abortus* stained with haematoxylin, the bacteria agglutinate rise up with the fat globules to form a deep blue ring in the cream layer. This test is very sensitive.

3.10 Antibiotic Sensitivity Tests

Antibiotic sensitivity tests are routine procedures in diagnostic bacteriology. These tests are performed to determine the sensitivity of a given bacteria to various antibiotics. The results of these tests are quite helpful to the clinician for the selection of a suitable antibiotic to treat the patient. It is desirable to collect the specimen before the administration of antibiotics. If cultures are done during antibiotic therapy, the causative organism may be inhibited due to the bacteriostatic or bactericidal action of antibiotics.

The pathogenic bacteria exhibit strain variations in susceptibility to antibiotics and chemotherapeutic agents. This is particularly true in the case of *Staph. aureus* and Gram negative bacilli like *Pseudomonas*.

There are two methods of performing this test:

I. Diffusion tests. II. Dilution tests: Broth dilution
Agar dilution

3.10.1 Diffusion Test

This is a simple, quick and widely used method. Here the isolated organism is inoculated into a broth and incubated for 6 hours in order to get the logarithmic phase of growth. This is quite essential, because the antibiotics are said to act on bacteria when they are in the logarithmic phase of growth. This 6 hours-old broth culture must be inoculated with the help of a sterile swab onto the surface of the nutrient agar so as to get a lawn culture. Then the drug has to be applied. There are several methods for the application of the drug.

3.10.1.1. Ditch Plate Method

Small wells are cut in the inoculated medium and are filled with different antibiotic solutions.

3 10.1 2 Cup Plate Method

Small, hollow, glass cylinders called *Heatley Cups* are slightly pressed over the inoculated medium The antibiotic solutions are added to these cylinders.

3 10 1.3 Disc Diffusion Method

This is the routinely employed method Filter paper discs 6 mm in diameter are cut using Whatman No 1 filter paper with a punching machine. These discs are sterilized in a hot air oven in penicillin bottles, each containing 100 discs. The antibiotic solutions are prepared so that 1 ml contains the amount required for 100 discs. i.e each disc will be charged with 0.01 ml of the antibiotic solution These charged discs are placed on the inoculated medium using sterile forceps

After the application of the drugs, the inoculated culture plates are incubated overnight at 37°C. If the inoculated organism is sensitive to a particular antibiotic, there will be a zone of inhibition of growth around the disc. If the organism is resistant to a particular drug, the growth will not be inhibited or there will not be any zone of inhibition around the disc. The diameter of the inhibition zone measured in mm and compared with that of the standard strain of organism represents the degree of sensitivitv.

The results of sensitivity tests are reported by referring to KIRBY-BAUER'S SENSITIVITY TABLE as 'Sensitive', 'Moderately sensitive', or 'Resistant' to the different drugs

Kirby-Bauer's Interpretative Sensitivity Chart

		Inhibition Zone Diameter in mm		
Drug	*Disc Concentration*	*Resistant*	*Moderately Sensitive*	*Sensitive*
Ampicillin	10 ugm	20 or less	21-28	29 or more
Chloramphenical	30 ugm	12 or less	13-17	18 or more
Erythromycin	15 ugm	13 or less	14-17	18 or more
Gentamycin	10 ugm	--	-	13 or more
Calistin	10 ugm	8 or less	9-10	11 or more
Kenamycin	30 ugm	13 or less	14-17	18 or more
Penicillin	10 units	20 or less	21-28	29 or more
Streptomycin	10 ugm	11 or less	12-14	15 or more
Sulfonamides	300 ugm	12 or less	13-16	17 or more
Tetracycline	30 ugm	14 or less	15-18	19 or more

3.10.2. Dilution Tests

3.10.2.1 Broth Dilution Method

Serial dilutions of the drug in broth are taken in tubes and a standardized suspension of the test bacterium is inoculated. After overnight incubation, the Minimum Inhibitory Concentration (MIC) is read by noting the lowest concentration of the antibiotic that inhibits the growth.

3.10.2.2. Agar Dilution Method

This is carried out in the same manner as above, but instead of liquid medium, agar media incorporated with different dilutions of antibiotics are used. The agar dilution method is used to determine the sensitivity pattern of *Mycobacterium tuberculosis*.

PART IV

IDENTIFICATION OF BACTERIA

4.1. Morphological Classification of Bacteria

Morphological classification of bacteria is based on the shape, characteristic arrangement or cell grouping and their reaction to the Gram or Ziehl-Neelsen stains. Depending on their shape, bacteria are distinguished into the following varieties:

1. *Cocci* - Spherical or nearly spherical cells
2. *Bacilli* - Relatively straight rod-shaped (cylindrical cells)
3. *Vibrios* - Curved rod-shaped cells (comma-shaped)
4. *Spirilla* - spirally twisted, non-flexous rigid rods
5. *Spirochaetes* are flexible spiral forms.

4.1.1. Cocci

The main groups of cocci are distinguished according to their mode of cell grouping and their reaction to the Gram stain. The different cocci are relatively uniform in size, about 1 μ in average diameter Some species are capsulated and a few are motile. A pure growth of cocci will show the predominant cell grouping arranged in clusters, or long chains and, at times, in singles, pairs, fours (tetrads), cubical packets, and very short chains.

1. *Streptococcus*: Cells mainly adherent in chains, due to successive cell division occurring in the same axis, Gram positive (e g. *Strepto pyrogene, Str. fecalis, Str. viridans).*

2. *Staphylococcus and Micrococcus*: Cells mainly adherent in clusters, due to successive divisions occurring in different planes, Gram positive (e.g.*Staph aureus*). Micrococci form irregular or rectangular clusters or tetrads or cubical packets (e.g. *Sarcena lutea Micrococcus tetragenous);* Gram positive.

3. *Neisseria*: Cells mainly adherent in pairs and slightly elongated at right angles to the axis of the pair; Gram negative (e.g. *N. meningitis*).

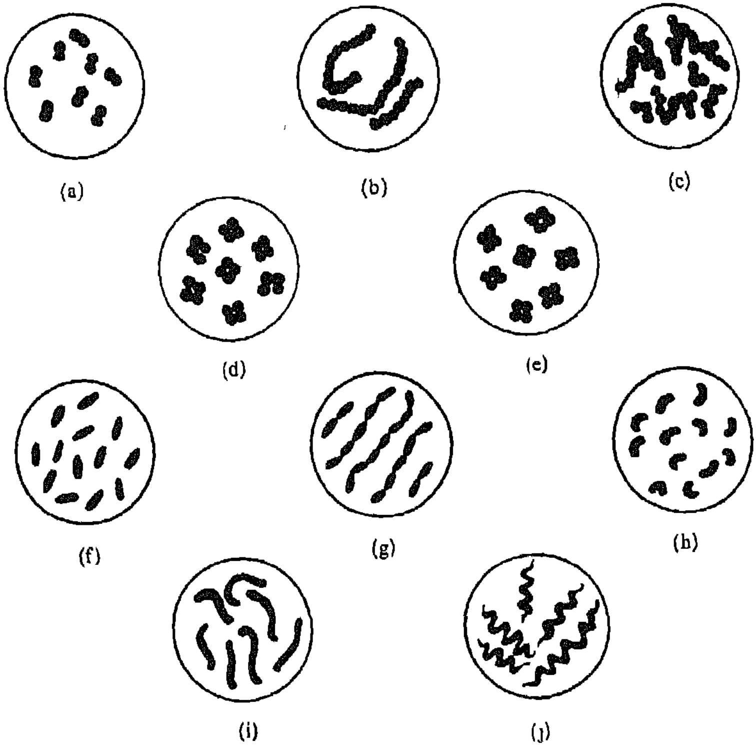


FIG 4 1

4. *Veillonella*· Generally very small cocci arranged mainly in clusters and pairs, Gram negative (e.g *Veill parvula*)

4.1.2. Bacilli

The primary subdivision of the rod-shaped bacteria i.e. bacilli, is made according to their staining reactions by Gram and Ziehl- Neelsen stains and whether or not endospores are formed Some of these groups include several biological genera and can be recognized only by a study of their physiological characteristics in artificial cultures

1. *Acid Fast bacilli*

In giving an acid fast staining reaction by the Ziehl-Neelsen method, members of the genus *Mycobacterium*, including the tubercular bacillus, are distinguished from all other bacilli (e.g. *Mycobac-*

terium tuberculosis, Myco leprae).

2. *Gram positive spore forming bacilli*

Except for some rare saprophytes, the only bacteria to form endospores are those of the genera *Bacillus* (aerobic) and *Clostridium* (anaerobic). They are primarily Gram positive but very liable to become Gram negative in old clusters. The size, shape and position of the spore may assist recognition of the species e g. the tetanus bacillus is characterized by its bulging, spherical, terminal spore ('drum stick' form) (e.g. *Clostridium welchii, Cl. tetani, Cl. botulinum*).

3. *Gram positive non-sporing bacilli*

These include several genera *Corynebacterium* is distinguished by a tendency to slight curving and club shaped or ovoid swelling of the bacilli and their arrangement in parallel and angular clusters due to the snapping mode of cell division (e.g *Corynebacterium diptheriae*)

4. *Gram negative bacilli*

These include numerous genera belonging to the families Pseudomonaceae, Enterobacteriae and Bacterioidaceae *Pseudomonas* is distinguished by its polar flagellation whereas mobile members of the other families are periotrichously flagellate (e.g *Pseudomonas aeruginora, Salmonella typhi, Shigella shigae*)

4.1.3 Vibrios and Spirilla

Vibrios are recognized as short curved rods (e.g. *Vibrio cholerae*) and spirilla as non-flexous spiral filaments (e.g *Spirillus*). They are Gram negative and mostly mobile, having polar flagella and showing very active darting mobility.

4.1.4 Spirochaetes

These microbes are like bacteria, but differ from bacteria by being slender, flexuous, spiral filaments. Their staining reaction is Gram negative. There are different varieties of spirochaetes and they can be recognized by their size, shape, wave form and refractility.

The pathogenic spirochaetes are classified in three genera.

1. *Borrelia*: They are large and more refractile than the other pathogenic spirochaetes and readily stained by ordinary methods The cells are large and open with a wave length of 2-3 (e.g. *Borr recurretin*).

2. *Treponema*: These are thin filaments in coils of shorter wave length (e.g. 1.0 - 1.5), typically presenting a regular 'cork-screw' form, feebly refractible and difficult to stain except by the silver impregnation method. (e.g. *Tr. pallidum*)

3. *Leptospira*: The coils are five and close (wave length about 0 5) and they are barely discriminable by dark ground microscopy One or both extremities of the spirochaete are 'hooked' or recurved, so that it may take the shape of a walking stick, and an S or a C.

4.2. Identification of Gram Positive Cocci

4.2.1. Gram Positive Cocci

The Gram positive cocci can be divided into two groups on the basis of the arrangement of their cells. cluster forming and chain forming. The catalose test also broadly supports the differentiation of the two groups, though there are a few exceptions. This test detects the presence of the enzyme catalose.

	Cluster forming cocci	*Chain forming cocci*
Cell arrangement	Grapelike clusters, tetrads, cubical packets; sometimes, short chains (less than 5 cocci in length)	Form chains The chain length is more than 5 cocci in length
Catalose test	Positive (except *aero coccus* which may be weak or negative)	Negative

Catalose test

For this, 3% solution of hydrogen peroxide is used, which is kept at 4°C.

For the test, the suspected colony, 18-24 hours old, is picked from the solid media on to a clean glass slide. A drop of hydrogen peroxide is put on it Production of gas bubbles from the organisms immediately (5-10 seconds) indicates catalose positive

Hydrogen peroxide also may be added over the colonies on solid media to see evolution of gas which also indicates catalose positive

Note.

1. Old colonies may give a false negative result
2. Hydrogen peroxide should be checked daily by testing with known catalose positive (Staph aureus) and negative (Strept-pyogenes) organisms as quality control.
3. Blood cells contain the enzyme catalose. For this reason, or-

ganisms grown on blood agar should be picked up carefully or, as far as possible, avoided, because this gives a false catalose positive test.

4. Iron wire loops also give rise to a false catalose positive test.

4.2.2. Gram Positive Cluster Forming Cocci

4.2.2.1 Staphylococcus

Morphology

These are Gram positive cocci about 1 μ in diameter, mainly joined in grape-like clusters. Cells may be found singly, in pairs, or short chains. They are non-sporing, non-motile and non- capsulate

Culture

After culture for 24 hours at 37°C on nutrient agar, milk agar or blood agar, the colonies are circular, 2-4 mm in diameter, with a smooth shiny surface. Pigmentation is a constant distinguishing feature in primary isolates of some staphylococci

Pigmentation	*Staphylococci*
Golden yellow	*Staphylococcus aureus* (*Staph pyogenes* var *aureus*, *Mycrococcus pyogenes* var *aureus*)
Lemon yellow	*Staph citreus*
White	*Staph albus*

The cocci grows well on basic media and also on McConkey's medium, although in the latter, the colonies are much smaller and appear deep pink in colour.

Biochemical reactions

The coagulase test is done to differentiate the pathogenic strain of this group from the non-pathogenic staphylococci. It depends on the production of an enzyme coagulase which coagulates blood plasma.

This test is done in two ways· the slide test and tube test.

Slide Coagulase Test

On a clean glass slide, a drop of normal saline is put. A suspension of staphylococcus colony is taken in a loop and mixed with the saline; minimum spreading is done to avoid drying. The suspension should look smooth or milky. (If it forms clumps, then it is self-agglutinable and the slide test is discarded.)

A sterile loop is dipped into the plasma at room temperature and the adhering traces of plasma mixed with the bacterial suspension. Within 5-10 seconds, the suspension forms a clumping which indicates that it is a positive reaction. In a negative reaction, the suspension remains uniform.

Tube Coagulase Test

About 1 ml of culture is placed in a sterile tube with an equal quantity of plasma (1/10 dilution) The tube is then incubated for at least four to six hours. If there is dot formation, then it is called a positive reaction and if the solution remains uniform, the result is negative

Note: The colony taken for the coagulase test should be pure and must be confirmed as Gram positive cocci and catalase positive, otherwise a false positive reaction may occur

The plasma clumping test is used for qualitative and routine purposes; the cocci are suspended in a drop of plasma and the coagulase positive strain clumps rapidly. The coagulase positive staphylococci are invariably pigmented. The coagulase positive strains are also haemolytic on blood agar.

Pathogenicity

Staphylococci are constantly present on the skin and in the upper respiratory tract. *Staph ameus* is the most virulent one It produces both superficial and deep pus forming infections It also causes food poisoning and enteritis. *Staph. epidermidis* causes urinary tract infection in some cases

4.2 2.2. Micrococci

Micrococci form irregular or rectangular clusters and tetrades of Gram positive cocci. Micrococci are less often isolated than staphylococci, and are rarely pathogenic. Most of them are saprophytic forms found in water and elsewhere in nature.

Sarcina lutea forms cubical packets of eight cells. It produces a bright yellow pigment

Micrococcus tetragenous (*Gaffkya tetrazene*) occurs in tetrades or groups comprising four small oval cocci. It produces a rough, elevated white growth on agar; liquefies gelatin; coagulates milk. Growth is slow at 20°C and 37°C. It occurs in the healthy human mouth and is of low-grade virulence.

4.2.3. Gram Positive Chain-forming Cocci

4.2 3.1. Streptococci

The streptococci are important human pathogens. Their infection has a tendency to spread unlike those of staphylococci which remain localized Differentiations of the streptococci on the basis of physiological and immunological methods have not been satisfactory. Preliminary separation of pathogenic streptococci is done on the basis of haemolysis and, thus, immunological methods are employed to define species and types.

Morphology

Streptococci are Gram positive cocci, mainly arranged in chains of different length. Each cell is approximately 1 μ in diameter. They are non-motile, non-sporing and may be capsulate.

Culture

On blood agar, they form pinpoint colonies with varying degrees of haemolysis.

They are roughly divided into three groups by their effect on blood agar.

(a) *Alpha Haemolytic*: There is a formation of green colouration of the medium around the colonies. The red blood cells are partially lysed (e.g. *Strep. viridans*).

(b) *Beta Haemolytic*. Here there is a formation of a clear zone of haemolysis around the colonies (e.g. *Strep. pyogenes*).

(c) *Gamma Haemolytic*. Here there is no obvious alteration of the medium around the colonies. There is no haemolysis (e.g *Strep. faecalis*).

In culture, they are small (1 μ in diameter), discrete, shiny, regular, and semi-transparent colonies. *Strep. pyogenes* and *Strep. viridans* require blood or serum for growth. But *Strep. faecalis* will grow easily both on nutrient agar and McConkey's medium.

The other means of differentiating *Strep. faecalis* from the other two is by heating a broth culture or suspension of the organism to 60°C for 30 minutes. Only *Strep. faecalis* will resist this heating and grow when subcultured.

Thus, differentiation methods are required to identify *Strep faecalis* from alpha and beta haemolytic *Streptococcus*.

However, the most reliable means of differentiating the beta haemolytic streptococci is by serological testing

Pathogenicity

Strep. pyogenes is a causative organism of sore throat, tonsilitis and scarlet fever. It also causes cellulitis and causes infection in burns.

Strep. viridans: It is a commensal of the intestine and occasionally causes urinary tract infection.

4.2.3.2 Pneumococcus (Streptococcus pneumoniae)

It is a Gram positive coccus about 1.0 μ diameter, ovoid or lanceolate, occurs in pairs with the broader ends together. Because of this it is also called *Diplococcus pneumoniae*. The virulent strains of diplococci are capsulated. In culture, they are usually arranged in short chains

Culture

After overnight incubation, the colonies appear similar to *Strep. viridans* and shows the same type of alpha haemolysis, but on further incubation, the centre and edge of the colony become ruined with concentric rings.

The organism is aerobic and requires the enrichment of blood or serus for growth

Biochemical Reaction

Pneumococci can be differentiated from *Strep viridans* by their solubility in bile or bile salts. When the bile or bile salt is added to a broth culture of the organism, lysis can be observed within 15 minutes

The test can be carried out by adding 0.1 ml of a 10% solution of sodium dichlorate to 5 ml of broth culture.

Pathogenicity

Pneumococci are the causative organisms of pneumonia and subacute bacterial endocarditis They also cause septicaemia

4.3 Identification of Gram Negative Cocci

The Gram negative cocci include gonococci and meningococci belonging to genus *Neisseria* and the anaerobic diplococci belonging to genus *Veillonella*

4.3.1. Neisseria

4.3 1.1 The Gonococcus (Neisseria gonorrhoeae)

The gonococcus has been termed *Micrococcus gonorrhoeae* and *Deplococcus gonorrhoeae*, but now it is more or less accepted that the bacterium is *Neisseria gonorrhoeae*

Morphology

The cocci occur in pairs in preparation made from gonorrhoeal pus, but in pure culture they appear as oval and spherical and often in irregular masses. They are differentiated from other cocci present in the urethral or vulvovaginal tracts through the staining characteristic of being Gram negative.

Culture

The cultural gonococci are identified with certainty compared to direct smear examination An infusion chocolate agar is the medium of choice. It is directly inoculated with the specimen (or sediment if it is urine or seminal fluid) The culture is incubated in 10% carbon dioxide. The oxidase-positive colonies are sub-cultured. Sugar fermentations in serum broth or serum agar with an indicator provide the basis for identification.

Pathogenicity

The gonococcus is the causative agent of gonorrhoeae.

4 3.1 2.The Meningococcus (Neisseria meningitidis)

The meningococcus has been variously designated as *Micrococcus meningitidis, M. intracellularis meningitidis* and *Neisseria intracel-*

lularis Both the terms, *N. intracellularis* and *N. meningitidis* are now used.

Morphology

The meningococcus is like the gonococcus and occurs in pairs or tetrads in direct film preparation of the exudate. In cultures, it appears in pairs, rarely as short chains. The coccus is non-motile and does not form spores.

Culture

Colonies in blood agar are moist, elevated and smooth. They have a bluish-gray tinge. They are differentiated from haemolytic and viridans streptococci and the pneumococcus by not producing green discolouration or haemolysis. The meningococcus can be differentiated from gonococcus by differential fermentation.

Pathogenicity

The meningococcus is the causative agent of cerebro-spinal fever or *cerebro-spinal meningitidis*.

4.3.1.3 The pigmented forms

The pigmented forms of *Neisseria* are found in the upper respiratory tract of man. They form a pale greenish-yellow pigment and are non-pathogenic. But *N. perflava* and *N. flava* have been found in meningitis and endocarditis. These Gram negative diplococci are differentiated from one another on the basis of fermentation.

Fermentation reaction of the Gram negative diplococci

	Non-pigmented		*Pigmented*	
	N. gonorrhoeae	*N. meningitidis*	*N Perflava*	*N flavescense*
Dextrose	+	+	+	-
Maltose	-	+	+	-
Sucrose	-	-	+	-
Levulose	-	-	+	-
Mannitol	-	-	+	-

4.3.2. Veillonella

Veillonella include obligate anaerobic Gram negative diplococci. They are smaller than *Neisseria*. They appear non-pathogenic but occur as parasites in the mouth, respiratory, intestinal and urogenital tracts. *Veillonella* include gas-forming (*V. parvula*) and non-gas-forming (*V. vulvovaginitidis*) groups.

4.4 Identification of Gram Positive Bacilli

Gram positive bacilli include the important pathogenic genera *Corynebacterium, Mycobacterium* and *Clostridium* The *Clostridium* contains anaerobic pathogens

4.4.1. Corynebacteria

These are Gram positive rod-like forms They are aerobic, non-motile, non-sporing, non-acid-fast, often staining irregularly and having a beaded appearance

4.4.1.1. Corynebacterium diptheriae

Corynebacterium diptheriae is the most important in this species and there are three types, *e g. gravis, intermedius* and *mitid*

Morphology

These are slender rods, with a tendency to show clubbing at both poles. The diphtheria bacillus ranges from 3-4 micron in length by 0.3-0.8 micron in thickness At the poles of the bacilli it has granules and special stains are used to stain these granules

Culture

Culturally, the corynebacteria are aerobes or facultative anaerobes, growing best at 37°C (range of 20°C to 40°C) on a blood or serum containing medium. Loeffler's serum slope and telluric medium are used for culture Colonies on Loeffler's medium appear at first small, circular, white, opaque discs with a regular border, later, the centres become thicker and the borders crenated.

Biochemical reaction

Fermentation reactions are important in differentiating the toxigenic strains from non-toxigenic strains The toxigenic strain produces acid from glucose and maltose but not as a rule from sucrose which is usually fermented by non-toxigenic strains.

Pathogenicity

Corynebacterium dipthenae is the causative organism of dipthena.

4.4.2. Mycobacterium

The mycobacteria or acid-fast bacilli have distinctive biological characteristics differentiating them from most other microorganisms. The mycobacteria are not easily stained but when stained with strong dyes resist decolourization with acid. The mycobacteria are thus called 'acid-fast' organisms. Another characteristic feature is the very slow growth of the pathogenic mycobacteria or even failure to grow e.g. *Mycobacterium leprae* on artificial culture media.

Mycobacterium are widely distributed throughout the world and only a few species are pathogenic to man and other mammals, birds, reptiles and fish. The pathogenic species that need particular attention are the *Mycobacterium tuberculosis* and *Mycobacterium leprae* which causes leprosy in man.

4.4.2.1. Mycobacterium tuberculosis (or Myco bovis)

Morphology

The two types of tubercle bacilli that ordinarily affect man i.e. the human type (*Myco tuberculosis*) and the bovine type (*Myco bovis*)

The human type i.e. *Myco tuberculosis* is a slender or slightly curved bacillus in direct smears measuring about 2.5 to 3.5 μ by 0.3 μ, although both shorter and longer forms may be seen. In cultures, short forms are often found, especially on solid media, but longer forms may be found in liquid media. The bacilli may occur singly or in pairs, in larger or smaller masses.

The tubercle bacillus does not stain easily by the ordinary dyes, but it stains well with a strong dye with a mordant, such as carbol fuchsin, when the stain is hot, but it takes a longer time when the stain is cold. Once it has been stained, it resists decolourization.

With 20% sulphuric acid or nitric acid; it also resists decolourization with alcohol and so it is both acid and alcohol fast The bacilli are Gram positive but by this method they are stained only with difficulty and Gram's method is of no use for this identification.

Culture

The tubercle bacilli will not grow on ordinary media. Primary cultures are usually made on some form of egg medium of which the Lowenstein-Jensen medium is the most widely used. Dorset's egg medium is an older medium and Petraguani's medium may also be used. After the primary culture has been established, it is possible to make subcultures on media without egg, such as Dubos, Proskaner and Beck, Kirschner and Youman's media.

The cultural characteristic of *M tuberculosis* is aerobic. It grows slowly. It does not grow on ordinary media but for the primary isolation requires an enriched medium, including egg, which is most commonly used.

On the Lewenstein Jensen medium, the general appearance of the growth is dry, irregular, tough, a buff to light orange in colour, but if the surface of the medium is moist, the appearance of the colony is smoother.

Biochemical reaction

Biochemical reactions are not ordinarily used in identification or classification of tubercle bacilli. Antigenic analysis has shown that there are four main serological groups of mycobacterium The human and bovine type of tubercle bacilli appear to be identical in antigenic composition, thus, explaining their cross reaction in the tubercular hypersensitivity test and the successful use of a bovine bacillus (BCG) vaccine for immunization against human type bacillus infection.

Pathogenicity

The *Mycobacterium tuberculosis* is the causative organism of tuberculosis in human beings

4.4.2.2. Mycobacterium leprae

The leprosy bacillus was recognized inside cells from leprosy nodules in 1874 by Hansen, a Norwegian doctor. It was the first bacterial pathogen to be described

Morphology

It is a straight or slightly curved, slender bacillus, about the same size as the tubercle bacillus, with pointed, rounded or club-shaped ends. It is non-motile and non-sporing. With the Ziehl-Neelsen stain, it is less strongly acid fast than the tubercle bacillus. The bacilli stain evenly in material from active lesions. The leprosy bacillus is Gram positive and can be stained fresh readily by Gram's method.

Culture

Until recently no claims were substantial but it is now believed that the organism may be isolated on the foot pads of mice.

Pathogenesis

M. leprae is the causative organism of leprosy. For diagnosis, smears are made from a scraping from the skin of suspected lesions and from nasal smears. It has also been found in sputum. The usual method is skin chips from the affected areas.

4.4.3. Clostridium — the Anaerobic Spore bearing Bacilli

The genus *Clostridium* comprises the Gram positive spore bearing anaerobic bacilli. Most species of this genus are saprophytes that normally grow in soil, water and decomposed plant and animal matter Some of them, e.g. *Clostridium welchii* and *Cl sporogenes*, are commercial inhabitants of the animal and human intestine. A few species are opportunistic pathogens and can produce disease. These include *Cl. welchii, Cl. septicum* and *Cl. oedematiens*, the cause of gas gangrene and other infections; *Cl. tetani*, the cause of tetanus; and *Cl botulinum*, the cause of botulism.

4.4.3.1. Clostridium tetani

Morphology

It is a Gram positive bacillus, 2-5 micron by 0.4-0.5 micron in dimension and possesses a terminal spore which is spherical Its appearance can be described as a "drum stick" appearance. *Cl tetani* is motile (20 to 30 peritrichous flagella).

Culture

Cl. tetani is an obligatory anaerobe. It is readily grown in cooked meat medium or in blood agar.

In blood agar under anaerobic conditions, *Cl tetani* grows and produces haemolysis In cooked meat medium, there is slight digestion and appears black.

Biochemical reaction

Cl. tetani does not ferment any sugar. The toxins of *Cl. tetani* are endotoxin and are extremely harmful to both man and animal

Laboratory diagnosis

The material is obtained from the wound.

(a) *Microscopical examination*. Films are made from the material directly and stained by Gram's method. If the presence of *Cl. tetani* is seen under the microscope, a culture must be done with the material for confirmation

(b) *Culture method*· The ideal medium for the purpose is cooked meat medium. Strict anaerobic conditions should be obtained. The cooked meat medium is heated to 75° - 80° for about 20 minutes to half an hour to kill the vegetative organisms Make smears from the culture, stain and examine for typical Gram positive bacilli with terminal and spherical spores.

(c) *Animal inoculation*: Toxin formation should be studied by culturing the organism in a suitable liquid medium and injecting the bacteria free filtrate into a susceptible animal (white mouse). This will develop the signs and symptoms of tetanus It is always advisable to use two animals, one with the toxin alone and the other which has been given a prophylactic dose of antitoxin

4 4 3 2 *Clostridium welchii*

Morphology

It is a long rod-shaped Gram positive bacillus forming a large oval central or subterminal spore It is non-motile

Culture

It grows in all the usual laboratory media and requires strict anaerobic conditions and prolonged incubation of up to 15 days. It grows best in carbohydrate-containing media like glucose agar or glucose blood agar On cooked meat medium, it produces invariant growth, producing gas and sour odour, and the meat turns pink in colour On blood agar, marked haemolysis is produced

Biochemical reactions

It ferments glucose, maltose, lactose and sucrose with formation of acid and gas

Pathogenicity

Cl welchii is the most important cause of gaseous gangrene It infects injured tissue and thus invades surrounding tissue

4 4 3 3 *Clostridium botulinum*

Morphology

It is a fairly large bacillus 0 5 to 0 8 by 0 3 to 8 0 microns with rounded ends, Gram positive, occurring singly, in pairs and in short or occasionally long chains It forms an oval terminal spore which is thicker than the bacillus. It is slightly mobile

Culture

It is strictly anaerobic. It grows well at a low temperature of $20^{\circ}C$ The optimum temperature is $35^{\circ}C$ The organism grows on ordinary media. In cooked meat media, it grows abundantly

Biochemical reactions

The Cl. botulinum is proteolytic and succhrolytic.

In cooked meat medium, vigorous growth takes place with the blackening of meat

Pathogenicity

This is the causative organism of botulism. Human botulism results due to eating badly preserved meat or imperfectly cooked meat

Laboratory diagnosis

Botulism is essentially a poisoning of the food The suspect food should be examined bacteriologically. It may be possible to demonstrate the presence of toxin in the patient's blood or in the post-mortem material. e.g. blood liver by direct animal inoculation. The gram-stained film of the food may first be examined for spore-bearing Gram positive bacilli.

4.5 Identification of Gram Negative Bacilli

Gram negative bacilli include a large number of bacteria comprising the following main groups

1. The coliform bacilli which include the commensal aerobic bacteria of the colon of man and animal
2. the Salmonella group which includes the typhoid, paratyphoid bacilli (of enteric fever) and the organism of bacterial enteritis or food poisoning
3. The Shigella or dysentery group

4.5.1. Salmonella

The Salmonella group comprising a large group of enteric bacilli are all pathogenic and include typhoid and paratyphoid bacilli.

Salmonella typhi

Morphology

It is a Gram negative non-sporing bacillus about 2-4 microns, actively motile with numerous long peritrichous flagella, as observed in special stained preparations.

Culture

It is an aerobe and facultative anaerobc; grows well on ordinary media at optimum temperature of 37°C; colonies on agar are like those of coliform bacillus but smaller, thinner and more transparent. No liquefaction of gelatin. Colonies on McConkey are smaller than those of coliform bacilli. Colonies are also pale or colourless on deoxycholate citrate medium.

Biochemical reactions

Ferments glucose and mannitol with production of acid, but no gas fermentation. Does not ferment lactose or sucrose and does not produce indole.

Salmonella paratyphi A and Salmonella paratyphi B

The morphology and general cultural characteristics are identical with those of typhoid bacillus. Important reactions are lactose — no change; glucose and mannitol — acid and gas; sucrose — no change. The typhoid bacillus does not produce gas while the paratyphoid bacilli produce gas.

Pathogenicity

Salmonella typhi, paratyphi A and *B* are the causative organisms of typhoid and paratyphoid fever in man.

The bacteriological diagnosis of typhoid fever depends on·

(1) The isolation from the body and the identification of the conservative organism;

(2) the demonstration of its presence in the body by the Widal agglutination reaction which is based on the occurrence of specific agglutinins to the organism in the serum of the infected person.

4.5.2. Shigella

The organisms of this group are the causative organisms of an acute form of dysentery, most prevalent in tropical countries. In cases like *Shigella shigae*, the disease may be virulent, characterized by high fever and tenemia. The stool is characteristically full of pus cells, without much blood or mucous. In severe cases, the stool resembles nothing but purulent material.

Shigella shigae (Shigella dysentriae) and Shigella flexner; (Shigella paradysenteriae)

Morphology

These are non-motile, non-sporing Gram negative bacilli about 2-4 micron by 0.5 micron in size. They often show tendency of shorter cocoon bacillary form.

Culture

They are similar to most other enterobacteria except that on McConkey or DCA medium they are lactose non-fermenting after incubation for 18 to 24 hours. Thus, on such differential media, colonies are 'pale' and similar to those of the other common pathogenic genus, Salmonella.

Biochemical reaction

The dysentery bacilli ferment glucose without gas production and in the case of sugar fermentation, generally are non-gas producing, H_2S is not produced.

Pathogenecity

The Salmonella group of organisms are responsible for acute bacillary dysentery in man

4.5.3. Pseudomonadaceae

They are Gram negative bacilli, which tend to elongate and some of these may produce a water soluble pigment The majority of these species are not pathogenic to man

Pseudomonas aeruginosa

Morphology

This is a Gram negative motile rod with 1-3 polar flagella, occurring singly, in pairs and chains

Culture

The colonies give a ground glass, blue-green appearance. *Pseudomonas* grows well on ordinary media

Biochemical reaction

This organism does not ferment lactose and since on McConkey agar the pigment may be masked, or absent, it may be confused with enteric pathogen pseudomonas. Strains isolated from clinical material are haemolytic in blood agar.

Pathogenicity

This organism is commonly found in air and water and is frequently found in urinary, ear and eye infections.

4.5.4. Vibrio cholerae (Spirillum cholerae, V. Comma)

Morphology

It is a bent or a slightly curved bacillus, resembling the shape of a comma. It is Gram negative and about 2μ in length. It is actively motile and the movement is of a darting or scintillating type This bacteria is also known as the common bacillus

Culture

It is aerobic and can grow slightly in anaerobic conditions also. It grows on ordinary media at a temperature range of 16-40°C. It grows abundantly on highly alkaline media of pH 8.2.

Colonies on nutrient agar are white, circular discs and transparent V cholera is non-haemolytic.

Biochemical reactions

It ferments glucose, sucrose, mannitose and maltose with only acid *V. cholerae* can produce indole and nitrites in peptone water.

Pathogenicity

Vibrio cholerae as the common bacillus is the causative organism of cholera. This disease is characterized by acute gastroenteritis of sudden onset and often running a fatal course.

4.5.5. Laboratory Identification of Gram Negative Bacilli Through Biochemical Reaction

Morphologically, the majority of Gram negative bacilli look alike and cannot be differentiated by mere microscopic or colony characteristic on a suitable medium. It is essential to differentiate the various species of these Gram negative bacilli in order to study their relationship with a particular disease, their incidence and prevalence in a particular area which helps in controlling or treating the infections caused by them.

A series of tests have been developed based on the biochemical and metabolic activities of these bacilli. These tests are being carried out routinely in the laboratory to identify the various species of Gram negative bacilli

For identification of Gram negative aerobic bacilli, the following tests are useful

(a) *Production of Indole* Indole is the breakdown product of the amino acid Tryptophan When an organism is grown in media containing Tryptophan (such as peptone water), it breaks down the Tryptophan producing indole This indole, when combined with a compound called para dimethyl, amino benzaldehyde (added in the form of Ehrlichi or Kova as a reagent) gives a pink coloured compound called Rose Indole.

Indole +ve Pink colour: e g : *Escherichia coli*

Indole -ve Yellow colour e.g.. *Klebsiella*

The test can also be conducted by inserting a paper (dipped in the reagent) at the mouth of test-tube containing overnight peptone water culture of the test organism

(b) *Methyl Red Test* Some Gram negative bacilli produce a large amount of acid in glucose phosphate broth The acidity can be determined by adding a few drops of 0.04% of methyl red solution as indicator. Red colour indicates acidity.

Red colour MR+ve e g : *Esch coli*

Yellow colour MR-ve e.g.: *Klebsiella*

(c) *Voges-Proskauer Test*: Some Gram negative bacilli produce a compound called Acetyl Methyl Carbinol from glucose This breakdown product, when it reacts with Alpha-Naphthol in alcohol, in the presence of alkali, gives a cherry red colour.

To overnight or 46-hour glucose phosphate broth culture add few drops of Alpha Napthol and few drops of 40% Potassium Hydroxide. Shake well and keep in the rack for 10 minutes.

Pinker cherry red colour VP+ve e g. *Klebsiella*

Any colour other than pink

or

Cherry red VP-ve e g. *Esch. coli.*

(d) *Urease production*: Some gram negative bacilli promptly decompose urea and liberate ammonia from it with the production of the enzyme urease. The production of the enzyme urease is detected by inoculating the organism in Christensep's urease agar (which contains urea, glucose, mineral salt and phenol red as indicator), when urea is split and ammonia is liberated, the phenol red turns pink due to alkaline pH.

Pink coloured agar slant with growth: Urease +ve e g *Proteus Klebsiella*

Yellow colour agar slant with growth. Urease -ve e.g.: *Esch coli*

(e) *Production of Hydrogen Sulphide*: Some Gram negative bacilli produce H_2S by breaking down aminoacids like cysteine or cystines This hydrogen sulphide production can be detected by exposing a filter paper dipped in 10% lead acetate solution:

— Blackening of the lead acetate paper

H_2S +ve: E.g.: *Citrobacter, Salmonella, Proteus*

— No blackening of the lead acetate paper

H_2S -ve: *Esch coli, Klebsiella*

(f) *Fermentation of Carbohydrates*. Many Gram negative bacilli are able to ferment a variety of carbohydrates, ultimately breaking them into acids like lactic acid, pyruvic acid, or into gases like CO_2 or hydrogen. The fermentation range of a particular organism is usually constant and rarely shows a variation In routine laboratory, various carbohydrates are used like.

Monosaccharides : Glucose,
Disaccharides : Lactose, Maltose
Polysaccharides : Starch, Inulin
Alkaloids : Salicin, Esculin
Polynhydric alcohols : Mannitol, Dulcitol, Inositol

Each carbohydrate is incorporated into peptone water to a concentration of 1% and an indicator called 'ANDRADES' indicator is added. (Acid fuchsin in alkaline solution) The medium is sterilized after distributing into sugar tubes with a small inverted glass tube called Durham's tube. The sugars are inoculated with the organism and incubated at 37°C for 24 hours. If the organism ferments a given carbohydrate due to acidity, the indicator turns pink. If it produces gas also, a bubble of gas is seen in the inverted Durham's tube placed inside the sugar tube. Results may be as given below

Pink colour with gas bubble in Durham's tube:	Carbohydrate is fermented with gas (Indicated by Symbol +)
Pink colour with no gas bubble in the Durham's tube:	Carbohydrate is fermented with acid production only but no gas (Indicated by the Symbol)
No colour change or yellow colour and no gas in the Durham's tube:	Carbohydrate not fermented (Indicated by the symbol –)
Organisms which ferment carbohydrates with acid and gas:	*Esch coli, Klebsiella, Citrobacter, Enterobacter.*
Organisms which ferment carbohydrates with acid only and no gas:	e.g: *Salmonella, Shigella, Vibrio.*
Organisms which do not ferment most of the carbohydrates.	e.g : *Pseudomonas, Alkaligenes.*

(g) *Utilization of citrate*: Some bacteria can utilize simple organic salts like sodium citrate or sodium acetate as a source of carbon and energy and are able to grow. During such utilization, CO_2 is liberated which makes the medium alkaline. Citrate utilization can be detected by inoculating the bacteria into Simmon's citrate or Kosar's citrate medium (sodium citrate, and mineral salts) Bromochymol blue as indicator) and incubating at 37°C for 24 hours.

Citrate utilized:	growth with deep blue slant	Citrate +ves e g.: *Klebsiella, Proteus*
Citrate not utilized.	growth with green slant	Citrate -ves e g.: *Esch. coli, Shigella*

IMViC Formula

Based on the production of *Indole, Methyl red test, Voges Proskauer test* (Vi stands for V-P, 'i' is included for the convenience of pronunciation) and *Citrate utilization*, some organisms can be identified. A positive reaction is indicated by + and a negative reaction by –

If we add up the reactions, the formula obtained indicates the IMViC reaction of a particular organism which helps in identification e.g.. IMViC formula for

Esch coli: ++–– Indicating that it is Indole

	and MR+ve but VP, Citrate - ve.
Klebsiella	- - + + Indicating that Indole, MR -ve but VP, Citrate +ve

All the above tests can be easily carried out and the results can be read in 24 hours Based on these tests, various species can be identified which helps in precise diagnosis and management of the infections caused by them

Biochemical Reaction of Gram Negative Bacilli

	H_2S	Dextrose	Lactose	Sucrose	Mannite	Sorbitol	Gelatin	Motility	Urease	Oxidase	Indole	Methyl Red	Voges-Proskauer	Citrate
Escherichia coli	-	G	V	V	+	+	-	+	-	-	+	+	-	-
Aerobacter aerogenes	-	G	V	+	+	+	-	+	-	-	-	-	+	+
Aerobacter cloacae	-	G	+	+	+	+	+	+	-	-	-	-	+	+
Klebsiella pneumonae	-	G	+	+	+	+	-	-	-	-	-	-	+	+
Salmonella typhi	+	A	-	-	+	+	-	+	-	-	-	+	-	-
Salmonella paratyphi A	-	G	-	-	+	+	-	+	-	-	-	+	-	-
Salmonella paratyphi B	+	G	-	-	+	+	-	+	-	-	-	+	-	+
Shigella dysenteriae	-	A	-	-	-	V	-	-	-	-	V	+	-	-
Shigella flexneri	-	A	-	-	+	V	-	-	-	-	V	+	-	-

Pseudomonas aeruginosa	–	a	–	–	–	+	+	–	+	–	–	–	+
Vibrio cholerae	–	A	–	+	+	+	+	–	+	+	+	–	+
Proteus vulgaris	+	g	–	+	–	+	+	+	–	+	+	–	V

A = acid
a = weak
G = acid and gas
g = acid and slight gas

V = variable
+ = fermentation or positive reaction
– = no reaction

4.6 Identification of Spirochaetes

Spirochaetes are spiral, elongated, motile, flexible organism. The three important genera are *Treponema, Leptospira* and *Borrelia.*

Morphology

They are exceedingly delicate, spiral filaments. 6-14 μ by 0.13 μ with six to twelve coils which are comparatively small, strong and regular. In the unstained condition the demonstration requires dark field illumination. *T. pallidium* cannot be demonstrated by the ordinary staining method. The organism may be demonstrated by Fontana's method or the India Ink method, using the exudate from the chancre.

Pathogenicity

T. pallidium is the causative organism of syphilis.

Diagnosis

It can be diagnosed by a serological test.

Appendix

Contributors

1. Dr. P.G. SIVANANDA
   Prof. and Head, Department of Microbiology
   Kasturba Medical College
   Manipal
   Karnataka
2. Dr. R.K. SHARMA
   Associate Professor
   D.M.S.
   All India Institute of Medical Sciences
   New Delhi 110029
3. Dr. S.K. GHOSH
   Department of Microbiology
   All India Institute of Medical Sciences
   New Delhi 110029.